
UNIVERSITY
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# UNDERSTANDING MICROBIAL SPOILAGE MECHANISMS OF MODIFIED ATMOSPHERE PACKAGED LIVE MUSSELS FOR QUALITY IMPROVEMENT AND SHELF-LIFE EXTENSION

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May 11, 2019

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**ABSTRACT**

Post-harvest conditions have a significant effect on the quality and shelf-life of seafood particularly live seafood such as mussels and oysters. The short shelf-life is a limiting factor for supplying live mussels to consumers. Currently, Tasmanian live mussels are distributed in modified atmosphere packaged (MAP) pouches with elevated oxygen concentration giving a shelf-life of 7-10 days. The main objective of this study was to understand the biological and physical mechanisms that lead to reduced product quality and spoilage of MAP live mussels (*Mytilus galloprovincialis*) to enable systematic improvements to be made.

In Chapter 2, the optimization of post-harvest storage conditions required for retaining freshness and quality of MAP live mussels was investigated. The impacts of storage temperature, initial headspace oxygen and duration of depuration on shelf life were determined in three phases. No commercial reference was used in phase 1 but was used in phases 2 and 3. The commercial packs were packed by and according to the company supplying the mussels used in this study. MAP live blue mussels were stored at the following temperatures (phase 1): 4˚C, 6.5˚C, 10˚C and 13˚C for 240 hrs, 120 hrs, 96 hrs and 78 hrs. Three concentrations of oxygen gas headspace composition: 100% $O_2$ (M100), 80% $O_2$ and 20% $N_2$ (M80) and 60% $O_2$ and 40% $N_2$ (M60) were investigated. The results showed that storage of MAP live mussels at 4°C with 80% initial oxygen in the headspace was most effective in terms of length of shelf-life. In phase 2, the headspace oxygen of the commercial packs reduced significantly from day 7 onwards. In phase 3, mussels were depurated for 4, 8 and 12 hr and packed at optimised headspace oxygen (80%) and stored at optimised temperature (4°C). This showed that 8 hr depuration of harvest mussels was more practically effective (requires less time) when compared with other treatments.

In Chapter 3, the microbial quality, freshness and volatile organic compounds (VOC) produced during storage of MAP live mussels at refrigerated temperature (4°C) were evaluated using headspace solid phase micro-extraction gas chromatography mass spectrometry (HS-SPME GC/MS). Multivariate statistical analysis was used to identify VOC contributing to either freshness or spoilage. The shelf-life of commercial packs (CP) and undepurated samples was found to be 7 days based on microbial counts and mortality; while that of depurated samples was 10 days. Freshness volatiles such as hexanal, heptanal, and octanal decreased with storage while spoilage volatiles such as dimethyl trisulphide, decanal, phenol and octadienol increased with storage. Dimethyl disulphide was more dominant in CP and undepurated samples than in depurated mussels and could be used as spoilage indicator.

In Chapter 4, 16S rRNA amplicon sequencing was used to describe spoilage microbial community succession in the mussel meat and pouch water of undepurated and depurated mussels of MAP live mussels stored at 4$^{o}$C. The microbial community diversity on days 7 and 10 were observed to be closely related. Spoilage microbiota became predominant mostly between day 7 and 10. Proteobacteria, Cyanobacteria and Firmicutes were the three major phyla observed in the mussel meat and pouch water of undepurated and depurated mussels. Only Proteobacteria were observed to be dominant in both mussel meat and pouch water of commercially packed mussels. Among these phyla, Cyanobacteria were more predominant on day 0 in mussel meat of

undepurated and depurated mussels while Proteobacteria were predominant in commercially-depurated mussels. Proteobacteria were dominant across the storage days in both undepurated and commercially packed mussels. They were dominant from days 0 – 10 in depurated mussels with Firmicutes becoming predominant by day 15). *Synechococcus* was dominant on days 0 – 7 in the mussel meat of undepurated mussels and days 0 -10 in depurated mussels. *Shewanella* was dominant on day 0 in commercially packed mussels. *Acidaminococcus* became dominant on day 10 in undepurated and day 15 in depurated mussels. However, *Psychrobacter* was observed to be dominant in commercially-depurated mussels on day 7 and further shift to *Acinetobacter* occurred by day 15. In the pouch water, *Acinetobacter* was dominant throughout the storage days in undepurated mussels while *Psychrobacter* was predominant in both depurated and commercially-depurated mussels. Commonly, *Acinetobacter* and *Psychrobacter* preceded *Shewanella* and *Acidaminococcus* as spoilage bacteria in live mussels.

In Chapter 5, the spoilage potential of specific spoilage bacteria in MAP live mussels was evaluated. A total of 46 $H_2S$-producing bacteria were isolated. Twenty-eight isolates were obtained from pouch water and 18 from mussel meat. The isolate with the highest enzymatic activities (protease, lipase and DNase) and $H_2S$ production was identified by 16S rRNA analysis as *Shewanella baltica*. An axenic culture of the isolate was inoculated into sterile mussel broth and incubated at 4°C for 10 days. Volatile metabolites produced during storage were evaluated with HS-SPME GC/MS. Dimethyl trisulphide, nonanal, decanal, phenol, pentyl furan 2 and octadienol were off-odour volatile metabolites produced by spoilage bacteria during storage of cooked mussels at 4°C for 10 days.

In Chapter 6, a Quality index method (QIM) tool that adapted the existing QIM concept and applied it to the assessment of MAP live mussels was developed in this study. The QIM tool consisted of 3 quality attributes with 7 parameters with 28 descriptors and up to 21 demerit points. A linear model was obtained that described the change in quality attributes (QI) with respect to storage days (QI = 1.261 x storage days - 1.6789, $R^2$ = 0.9546, n = 3). The total viable count (TVC), mortality and headspace $CO_2$ all increased with storage days while headspace oxygen decreased. The shelf-life was estimated as 10 days based on acceptance rejection (due to objectionable smell) by the assessors as well as microbial and biochemical assessment. The QIM tool developed could be used to assess the freshness and acceptance of live mussels.

In conclusion, inadequate duration of depuration, pouch water and low headspace oxygen in the gas atmosphere contribute to the spoilage of live mussels. Inadequate duration of depuration likely enhances succession of spoilage bacteria by leaving a higher residual microbial population, while pouch water provided the biological medium for extensive microbial growth. Microbial respiratory consumption of the reduced headspace oxygen may stress the mussels which may result in mortality and evolution of volatile compounds that led to objectionable smell in MAP live mussels.

**STATEMENT OF CO-AUTHORSHIP**

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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| | | |
|---|---|---|
| AFLP | - | Amplified fragment length polymorphism |
| ARISA | - | Automated ribosomal intergenic spacer analysis |
| CFC | - | Cephaloridine fucidin cetrimide (CFC) agar |
| CFU | - | Colony forming unit |
| DMA | - | Dimethylamine |
| DNA | - | Deoxyribonucleic acid |
| EMA | - | Ethidium monoazide |
| ERIC-PCR | - | Enterobacterial repetitive intergenic consensus |
| FFA | - | Free fatty acid |
| $H_2S$ | - | Hydrogen sulphide |
| HCN | - | Hydrogen cyanide |
| IA | - | Iron Agar |
| LAMP | - | Loop mediated isothermal amplification |
| LH | - | Long and Hammer Agar |
| MA | - | Marine Agar |
| MALDI-TOF MS | - | Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry |
| MAP | - | Modified atmosphere packaging |
| MTA | - | Methylamine |
| MRS | - | De Man, Rogosa and Sharpe agar |
| NO | - | Nitric oxide |
| PCA | - | Plate count agar |
| PCR | - | Polymerase chain reaction |
| PCR-DGGE | - | Denaturing gradient gel electrophoresis |
| PCR-TTGE | - | PCR - Temporal Temperature Gel Electrophoresis |
| PFGE | - | Pulsed-field gel electrophoresis |
| QIM | - | Quality index method |
| RAPD-PCR | - | Randomly amplified polymorphic DNA PCR |
| REP-PCR | - | Repetitive element palindromic PCR |
| RNA | - | Ribonucleic acid |
| SSO | - | Specific Spoilage Organisms |
| STAA | - | Streptomycin Thallium Acetate Agar |
| ssPCR | - | Species Specific Polymerase Chain Reaction |
| TMA | - | Trimethylamine |
| TMAO | - | Trimethylamine-oxide |
| TRFLP | - | Terminal restriction fragment length polymorphism |
| TSA | - | Tryptone Soy Agar |
| TVBN | - | Total volatile base nitrogen |
| TVC | - | Total viable count |
| VOC | - | Volatile organic compounds |
| VRBGA | - | Violet Red Bile Glucose agar |

# Chapter 1: General introduction

## 1.1 Introduction

Seafood such as bivalve molluscs, finfish and crustaceans serve as a major source of income in many countries (FAO, 2017). Bivalve molluscs such as mussels, oysters, clams and scallops are characterized by a sedentary filter-feeding nature and their filter-feeding capacity can result in the concentration of marine bacteria. Therefore, harvested bivalves need to be properly processed to reduce the microbial load of indigenous and contaminating bacteria. Improper post - harvest handling, water activity, packaging methods and storage temperatures could increase indigenous bacterial numbers that cause seafood spoilage (Huss et al., 2000). Seafood is highly perishable and is termed spoiled when it becomes unpalatable and unacceptable due to microbial, autolytic or enzymatic activities. Due to the scope of this thesis, only microbial spoilage will be further discussed.

### 1.2.1 Microbial spoilage

Microbial spoilage of seafood results in a huge economic loss (Gram and Dalgaard, 2002). Total viable counts (TVC) involving culturing of bacteria on general agars have been used as an internationally accepted standard quality indicator in seafood (Dalgaard, 2000). Not all enumerated microbes are involved in seafood spoilage, but typically, only a few of the Gram-negative normal flora known as specific spoilage organisms (SSO). The spoilage of seafood results in off - odours (volatile organic compounds/metabolites - VOC ) (Figure 1.1) (Gram and Dalgaard, 2002; Kuuliala et al., 2018) thereby reducing the shelf-life.

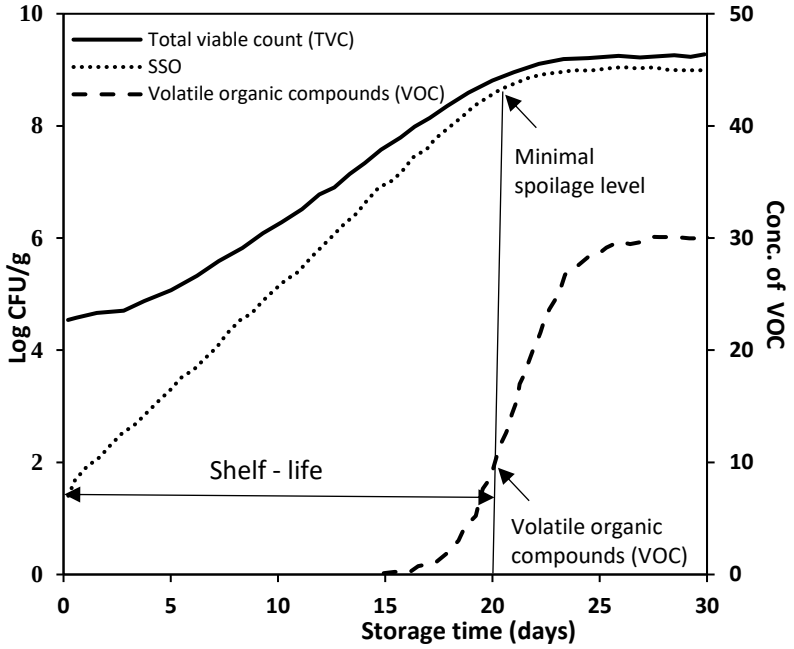


**Figure 1.1:** Microbial growth and spoilage of seafood. Adapted from Gram (1989).

In providing an overview of microbial and biochemical food spoilage, Huis in't Veld (1996), stated that spoilage bacteria are usually present in small numbers, but increase with increased storage days and under storage conditions such as increased temperatures (Huis in't Veld, 1996). The knowledge of SSO present in seafood could help in shelf-life determination, its prediction and extension (Dalgaard, 2000). Dominant SSO in seafood are further described below.

#### 1.2.1.1 *Shewanella*

The genus *Shewanella* comprises of Gram-negative, motile, rod-shaped, glucose non-fermenting, oxidase positive, catalase positive bacteria that are members of the family *Shewanellaceae,* mostly found in aquatic environments (Satomi et al., 2007). Members of

the genus such as *Shewanella putrefaciens, Shewanella baltica*, also known as *Shewanella liquefaciens*-like are psychrotolerant and capable of producing $H_2S$ in low storage temperatures (Zhu et al., 2015). Members of this genus have been isolated from cold smoked salmon (Joffraud et al., 2006), shrimps (Jaffres et al., 2009), cooked shrimps (Mace et al., 2014), oysters (Richards et al., 2008) and raw fish stored on ice (Fonnesbech et al., 2005). In a recent study, both *S. baltica* and *S. putrefaciens* were the predominant spoilage species at the end of the shelf-life of refrigerated yellow croaker (Zhu et al., 2016). They can also cause spoilage of seafood stored under vacuum packaging (VP) or modified atmosphere packaging (MAP) conditions (Gram and Melchiorsen, 1996).

Characteristically, seafood spoilage members of the genus *Shewanella* produce trimethylamine and dimethylamine compounds as off-flavours (Satomi et al., 2007). The ability of this genus to grow on Iron agar with characteristic black colouration makes it easy to isolate and identify. *Shewanella* produced 3-methyl-1-butanol, and 1-butanol as VOC in modified atmosphere packaged – MAP ($CO_2/O_2/N_2$: 60%/10%/30%) sterile fish substrate stored at 0 and 15 °C (Parlapani et al., 2017).

#### 1.2.1.2 *Pseudomonas*

*Pseudomonas* are aerobic, non-fermentative, oxidase positive, $H_2S$-producing motile Gram-negative bacteria (Lunestad and Rosnes, 2008; Parlapani et al., 2015). Members of the genus *Pseudomonas* that are commonly detected and associated with seafood spoilage include *Pseudomonas fragi, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas vranovensis* (Mace et al., 2012). *Pseudomonas* spp. can be isolated from homogenised

samples of seafood with cetrimide-fucidin cephaloridine agar (CFC) incubated for 48 hours (Miks-Krajnik et al., 2016). They have been isolated as spoilage bacteria from mussels (Aru et al., 2016b) and other seafood such as catfish fillet (Nguyen et al., 2014; Pang et al., 2015) and chill-stored sea bream (Parlapani et al., 2014), and gilt-head sea bream (Tryfinopoulou et al., 2002).

*Pseudomonas* spp. can inhibit each other in a seafood matrix. For example, Parlapani and Boziaris (2016) observed that *P. fluorescens* outcompeted *P. fragi* at higher storage temperatures such as 15°C. *Pseudomonas* spp. caused spoilage of raw salmon under aerobic conditions (Mace et al., 2012). They outgrew other spoilage bacteria in fish samples stored at 0˚C and 5˚C (Parlapani and Boziaris, 2016) due to faster growth (Broekaert et al., 2013) at temperatures above 4$^{o}$C (Pang et al., 2015). Mace et al. (2012) observed that *Pseudomonas* spp. dominate spoilage of MAP raw salmon at the onset of storage at 2°C – 8°C, but could not be detected on day 7, as $CO_2$ inhibited *Pseudomonas* spp. in MAP seafood (Gram and Huss, 1996; Mastromatteo et al., 2010; Reynisson et al., 2009). *Pseudomonas* spp. produced 3-methyl-1-butanol, 2-ethyl-1-hexanol and 3- methyl butanal as spoilage VOC in raw salmon stored at 4°C for 14 days (Miks-Krajnik et al., 2016).

#### 1.2.1.3 *Photobacterium*

*Photobacterium* a marine, motile, psychrotrophic, facultative Gram-negative genus, is similar to *Shewanella* due to the production of $H_2S$, being a non-sugar fermenter, oxidase positive and indole negative (Richards et al., 2008) but is a member of *Vibrionaceae* family (Jerome et al., 2016). The genus comprises of *Photobacterium phosphoreum*, *Photobacterium*

*iliopiscarium, Photobacterium aquimaris, Photobacterium piscicola* and *Photobacterium kishitanii* (Figge et al., 2014). However, *P. phosphoreum* is more associated with seafood spoilage than the others (Mace et al., 2013b). The inability to develop selective growth agar for *Photobacterium* makes it difficult to detect in seafood thereby making it difficult to control (Macé et al., 2013a; Mace et al., 2013b). The bioluminescent nature of this genus makes it easy to identify on agar plates such as Long and Hammer agar when viewed in the dark, however, bioluminescence can easily be lost after three days thereby making it difficult to identify colonies (Figge et al., 2014; Flodgaard et al., 2005). In a few studies, a conductance-based method has been used to enumerate this genus in seafood, especially *P. phosphoreum* (Dalgaard, 1995b; Emborg et al., 2002). *P. phosphoreum* has been detected in several studies as a spoilage bacterium in seafood such as salmon (Mace et al., 2014). It was the main spoilage organism in lobster (Gornik et al., 2011), and shrimp (Mace et al., 2013b) because it can survive at high $CO_2$ (Gornik et al., 2013).

*P. phosphoreum* is sensitive to heat, only being able to grow at < 25°C and requires sodium chloride (NaCl) for growth thereby making it difficult to use culture dependent methods to enumerate this bacterium in seafood (Timo et al., 2016). According to Timo et al. (2016), *P. phosphoreum* produces various spoilage VOC in seafood such as diacetyl, isobutyraldehyde, acetic acid, ethyl acetate, butanal, 2-methyl, butanal, 3-methyl, 1 propanol-2 methyl; 3-methyl-2-butanol, benzaldehyde and benzene acetaldehyde. In a recent study, *P. phosphoreum* was used to predict seafood shelf life at the cut-off level of 6.5 log CFU $g^{-1}$ and was found to inhibit growth and survival of other spoilage bacteria such as *Shewanella* and *Brochothrix thermosphacta* in raw salmon (Miks-Krajnik et al., 2016). In a study by Dalgaard

(1995b), it was observed that *P. phosphoreum* was capable of producing far more trimethylamine (TMA) during spoilage of fish than *S. putrefaciens*.

#### 1.2.1.4 *Brochothrix*

*Brochothrix thermosphacta*, a psychrotrophic non-motile, halophilic, non-sporulating, rod shaped Gram-positive bacterium, can grow within a wide range of temperatures (Mamlouk et al., 2012; Nowak et al., 2012). The bacterium is able to grow under aerobic and anaerobic conditions (Casaburi et al., 2015). It has been detected in packaged raw or cooked seafood. (Drosinos and Nychas, 1996; Drosinos et al., 1997; Fall et al., 2010; Jaffres et al., 2009). *B. thermosphacta* is resistant to Streptomycin hence, it is usually detected in seafood using streptomycin sulphate thallous acetate agar (STAA). Under aerobic conditions, *B. thermosphacta* produces caramel off-odours (2,3-butanedione) in seafood (Laursen et al., 2006). Fall et al. (2012) reported that *B. thermosphacta* produced spoilage VOC such as 2-pentanone, 3-hydroxy-2-butanone, 2-pentanol, 3-methyl-1-butanol, 4-methyl-3-chloro-2-pentanol and 2,3 butanedione from tropical cooked peeled shrimp during storage at 8°C for 14 days.

#### 1.2.1.5 *Psychrobacter*

The genus *Psychrobacter* consists of Gram-negative, psychrotrophic, non-motile, oxidase positive, rod shaped, aerobic and osmotolerant bacteria mostly found in seafood (Bowman, 2006). Members of this genus include *Psychrobacter immobilis, Psychrobacter cibarius, Psychrobacter maritimus, Psychrobacter proteolyticus* and *Psychrobacter fozii* (Broekaert

et al., 2011) and they have been found in seafood such as mackerel, angler fish, lobster, oysters and Atlantic cod (Broekaert et al., 2011; Broekaert et al., 2013; Meziti et al., 2010).

*Psychrobacter* species, especially *P. immobilis*, are capable of breaking down lipids and hydrolysing amino acids thereby causing slightly fishy and musty off-odours (Broekaert et al., 2013; Prapaiwong et al., 2009). Common spoilage VOC produced by this genus in sterile shrimp stored at 4 °C included 2, 3-dimethyl-oxirane, 2-butanone, 2-formylhistamine, 2-methyl-2-propanol, acetaldehyde (Broekaert et al., 2013).

#### 1.2.1.6 *Pseudoalteromonas*

The genus *Pseudoalteromonas* consists of aerobic, single-polar flagellated, rod -shaped, heterotrophic, halophilic, oxidase and catalase positive, non-glucose fermentative, heat-labile Gram-negative bacteria and is mostly found in the marine environment (Al Khudary et al., 2008; Broekaert et al., 2011; Oh et al., 2011). The genus consists of 39 species and two subspecies (Broekaert et al., 2011; Matsuyama et al., 2013; Oh et al., 2011). Members of this genus have been isolated on marine agar from sources such as seawater, sediment and seafood (Matsuyama et al., 2013; Ying et al., 2016; Zhao et al., 2014). They produce enzymes such as lipase (Xu et al., 2010), chitinase, agarase, amylase and protease (Ivanova et al., 2003). They have been associated with seafood spoilage recently. For example, *Pseudoalteromonas elyakovii, Pseudoalteromonas paragorgicola* and *Pseudoalteromonas nigrifaciens* dominated cooked shrimp under aerobic storage (Broekaert et al., 2013). Similarly, *Pseudoalteromonas* spp. were associated with spoilage of shrimp due to the ability to break down lipids, hydrolyse amino acids and protein (Broekaert et al., 2013). *P.*

*nigrifaciens* and *P. elyakovii* were detected in both peeled and unpeeled shrimp stored on ice and at 4$^{o}$C or 7$^{o}$C while *Pseudoalteromonas translucida, Pseudoalteromonas arctica and Pseudoalteromonas fuliginea* were dominant species in unpreserved unpeeled shrimps stored on ice (Broekaert et al., 2013). Broekaert et al. (2013) observed that *Pseudoalteromonas* spp., inoculated into sterile shrimp stored at 4°C produced the following spoilage VOC when the cells exceeded 8 log CFU $g^{-1}$: 1-methoxy-butane 1, 2-methylfuran, 2-butanone, 2, 3-dimethyl-oxirane cis, 2-butanediol, 2-propanol, 2-ethoxy-propane, 2-pentanone, 2-formylhistamine, isopropyl alcohol, 2-methyl-propanol, trimethylamine and 1, 2-butanediol.

#### 1.2.1.7 *Carnobacterium*

The genus *Carnobacterium* consists of non-motile lactic acid bacteria and some were shown to be bio-protective bacteria in seafood, but others were later observed to be the dominant spoilage bacteria in chilled vacuum packaged or modified atmosphere packaged seafood (Dalgaard et al., 2003; Laursen et al., 2005; Mace et al., 2012). Among the species of this genus, *Carnobacterium maltaromaticum*, *Carnobacterium divergens, Carnobacterium jeotgali, Carnobacterium iners, Carnobacterium mobile* have been associated with spoilage of seafood (Calliauw et al., 2016; Jaffres et al., 2009; Laursen et al., 2006). Isolation of *Carnobacterium* from seafood is mainly through the use of de Man, Rogosa, and Sharpe agar (MRS) but recently, Calliauw et al. (2016) were able to isolate *C. jeotgali* and *C. maltaromaticum* from plate count agar (PCA). However, the acetate in MRS could limit the growth of *Carnobacterium* (Leisner et al., 2007). Jaffres et al. (2011) observed that *C. maltaromaticum* was among spoilage bacteria isolated from shrimp stored for 27 days at

8°C. Dalgaard et al. (2003) isolated *Carnobacterium* as part of the spoilage microbiota from modified atmosphere packaged cooked and brined shrimps at low storage temperatures. However, it has been observed in recent studies that *Carnobacterium* could be dominant spoilage bacteria in seafood (Jaffres et al., 2009; Noseda et al., 2012). Calliauw et al. (2016) reported that on day 7, *Carnobacterium* spp., were the dominant spoilage bacteria in peeled brown shrimp stored at 4°C. Similarly, they enhanced rapid spoilage of shrimp and production of volatiles such as 1-pentanol, methyl mercaptan and 3-hydroxybutanone (acetoin) as reported by Mace et al. (2014) who investigated spoilage of cooked whole tropical shrimp.

### 1.2.2 Methods of microbial assessment of seafood

#### 1.2.2.1 Microbiological methods

##### 1.2.2.1.1 Culture-dependent methods

Culture-dependent methods or traditional methods have been used for several decades to study seafood spoilage microbiota (Table 1.1). They involve the use of media (selective, differential and general media) to culture and isolate bacteria from seafood (Amann et al., 1995). Thereafter, biochemical characterization and possibly typing are carried out to identity the isolates to species or even strain levels. Although culture-dependent methods are usually inexpensive and simple, there are limitations (Law et al., 2014; Madigan et al., 2013; Madigan et al., 2012). Most importantly, culture methods only show what can grow on the agar thereby underestimating the microbial community, diversity and predominance.

Other limitations of culture methods are: (1) they are time-consuming (Madigan et al., 2012; Madigan, 2014); (2) they are laborious; (3) unable to detect non-culturable bacteria; (4) a lack of growth factors required by some bacteria; (5) a lack of reproducibility, sensitivity and limited output information (Kim et al., 2014) and (6) they only reveal viable bacteria and not the total (viable unculturable) bacteria (Lee and Levin, 2007). They are time-consuming because some bacteria require pre-enrichment before growing in selective media and this may extend the procedure to more than 3 days to complete the isolation of the bacteria, the biochemical characterization and the molecular confirmation (Figure 1.2).

**Table 1.1:** Techniques used in studies of bacterial community succession in seafood

| Technique | Seafood | Bacteria | References |
|---|---|---|---|
| **Culture dependent** | | | |
| **PCA, MA, IA, MRS STAA, VRBGA, TSA, LH** | Peeled cooked brown shrimp, oysters, fish, Peeled cooked tropical shrimps, cold smoked salmon, fish fillet, raw salmon, catfish, sea bass, sea bream | *Arthrobacter bergerei, Shewanella putrefaciens, Brochothrix thermosphacta, Vagococcus salmoninarum, Shewanella* spp., *Pseudomonas* spp., *Psychrobacter* spp., *Carnobacterium* spp., *Serratia liquefaciens, Buttiauxella noackia, Escherichia coli, Enterococcus* spp., *Serratia phymutica, Enterobacter agglomerans, Hafnia alvei, Acinetobacter baumanii, Aeromonas salmonicida* | Calliauw et al. (2016); Garcia et al. (2015); Cruz-Romero et al. (2008); Jaffres et al. (2009); Jorgensen et al. (2000); Miks-Krajnik et al. (2016); Madigan et al. (2014); Milne and Powell (2014); Parlapani et al. (2014); Fidalgo et al. (2018) |
| **Culture independent** | | | |
| **PCR-DGGE** | Raw lobster tails, whole lobster, oyster | *Psychrobacter* spp., *Pseudoalteromonas* spp., *Pseudomonas* spp., *Luteimonas* spp., *Aliivibrio* spp., *Psychrobacter* spp., *Planococcus* spp., *Exiguobacterium* spp., *Carnobacterium* spp., *Pseudomonas* spp., *Chryseobacterium* spp. | Bekaert et al. (2015); Chen et al. (2013) |
| | Fresh shrimp | *Staphylococcus* spp., *Carnobacterium* spp., *Shewanella* spp., *Psychrobacter* spp., *Carnobacterium* spp., *Psychrobacter, Brochothrix, Shewanella* spp. | Broekaert et al. (2011); Broekaert et al. (2013) |
| | Peeled, cooked brown shrimp Farmed Atlantic cod | *Pseudomonas* spp., *Photobacterium* spp., *S. putrefaciens and Pseudomonas* spp., *Photobacterium phosphoreum, Pseudomonas* spp., *Shewanella baltica, S. putrefaciens* | Hovda et al. (2007) |

**Table 1.1:** Techniques in monitoring bacterial community succession in seafood (Continued)

| Technique | Seafood | Bacteria | References |
|---|---|---|---|
| **Next-generation sequencing (NGS), Pyrosequencing** | Peeled cooked, brown, shrimp half shell Pacific oysters | *Carnobacterium* spp., *Psychrobacter* spp., *Shewanella* spp., *Aeromonas* spp., *Chryseobacterium* spp., *Flavobacterium* spp., *Prosthecomicrobium* spp., *Arcobacter* spp., *Pseudoalteromonas spp., Vibrio* spp., *Helicobacter* spp., *Terasakiella* spp. | Calliauw et al. (2016); Madigan et al. (2014); Milne and Powell (2014); Rong et al. (2018) |
| **ssPCR** | Refrigerated seafood products, shrimps, cold smoked salmon raw salmon, sea beam | *Streptococcus parauberis, Vagococcus penaei, Serratia proteamaculans, P. phosphoreum, Brochothrix thermosphacta, Yersinia intermedia, H. alvei, Carnobacterium maltaromaticum, Acinetobacter baumanii, A. salmonicida* | Jaffres et al. (2010); Fernandez-No et al. (2012); Parlapani et al. (2013a) |
| **MALDI-TOF MS** | Seafood products, Tropical cooked and peeled shrimps, raw salmon | *Streptococcosis parauberis, Serratia liquefaciens, B. thermosphacta, Enterococcus faecalis, C. divergens, C. maltaromaticum, P. phosphoreum* | Jaffres et al. (2009); (Mace et al., 2012) |
| **SDS–PAGE** | Vacuum-packed salmon | *Chryseobacterium piscicola, P. phosphoreum* | Jerome et al. (2016) |
| **AFLP** | Salmon products | | |

PCA = Plate Count Agar; MA = Marine Agar; IA = Iron Agar; LH = Long and Hammer Agar; MRS = de Mann, Rogosa, Sharpe agar; STAA = Streptomycin Thallium Acetate Agar; VRBGA = Violet Red Bile Glucose agar, TSA = Tryptone Soy Agar; PCR-TTGE = Polymerase Chain Reaction - Temporal Temperature Gel Electrophoresis; PCR DGGE Denaturing gradient gel electrophoresis; ssPCR = Species Specific Polymerase Chain Reaction, MALDI-TOF MS = Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; AFLP = Amplified fragment length polymorphism; SDS-PAGE = Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

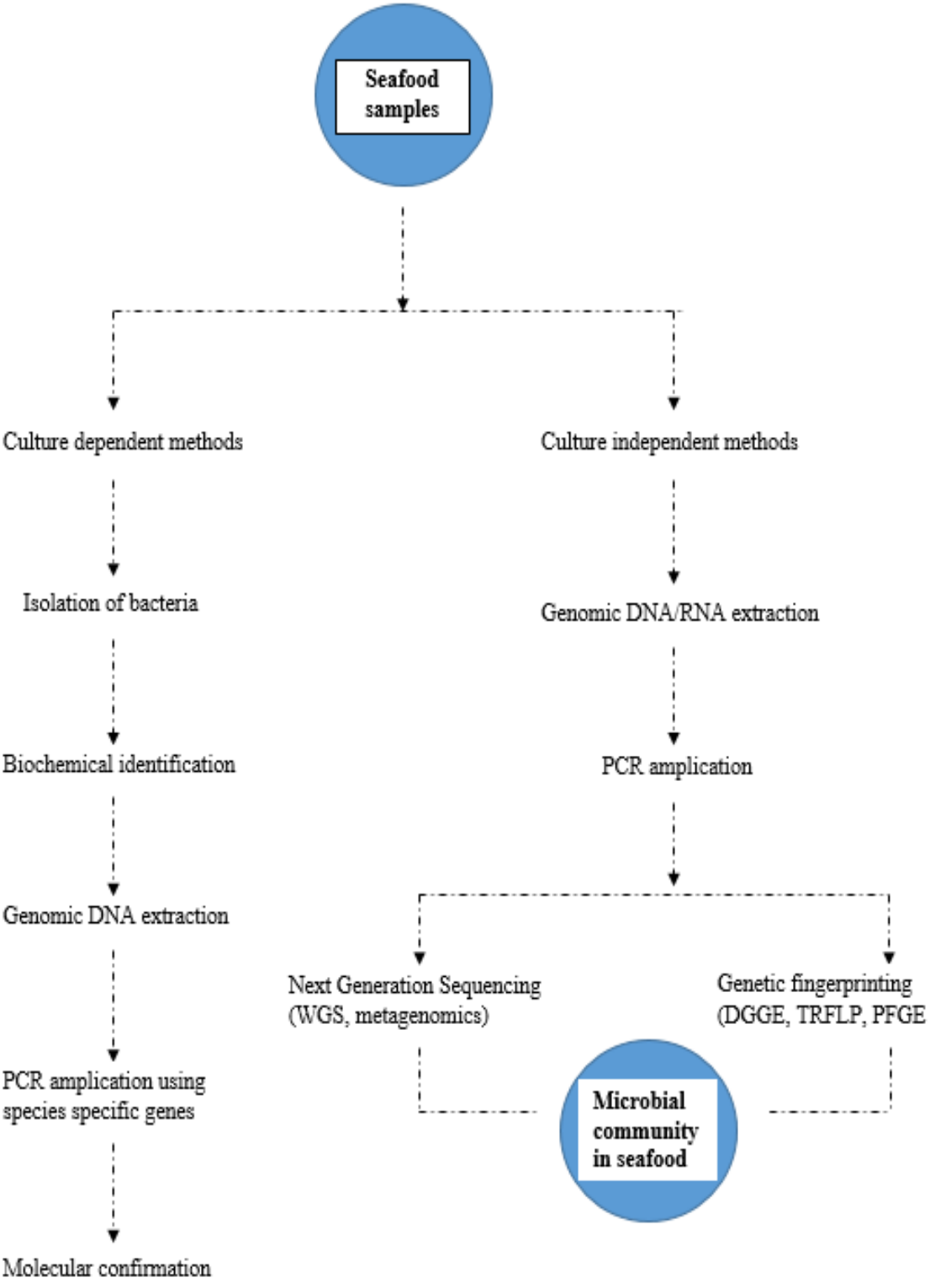


**Figure 1.2:** Flow chart of culture dependent and culture independent methods of identification of seafood spoilage bacteria.

The risks involved in the preparation and autoclaving of culture media and cross contamination of plates also limits the effectiveness of culture dependent methods (Zhao et al., 2014). Detection of bacteria present in low numbers is very difficult due to overgrowth by more common species and the presence of inhibitory substances in the samples (Naravaneni and Jamil, 2005). Bacteria such as *Vibrio, Listeria monocytogenes* and *Aeromonas* could give false negative result due to the ability of these bacteria to be viable but non-culturable (Law et al., 2014).

Growth media are not suitable for enumeration of some seafood spoilage bacteria such as *P. phosphoreum* (Mace et al., 2013b). It may take several days to identify the spoilage bacteria in seafood using culture-dependent methods. To overcome these limitations, there was a need to develop rapid culture-independent methods that can help in early, sensitive, highly informative and timely detection of seafood spoilage communities. This would then help to develop appropriate intervention strategies to restrict spoilage.

#### 1.2.2.1.2 Culture-independent methods

Culture-independent methods, also known as molecular methods, involve the use of polymerase chain reaction (PCR) - based techniques to study microbial communities in different food samples, including seafood, by analysing deoxyribonucleic acid (DNA) and / or ribonucleic acid (RNA) sequences which could be (Bhowmick et al., 2012; Mayo et al., 2014). Mostly, culture independent methods target amplification of the hypervariable V3-region of the 16S rRNA gene with universal primers and could be used to identify both cultivable and

uncultivable bacteria (Hovda et al., 2007). These methods involve the following steps: extraction of DNA/RNA from seafood samples or isolates from culture plates; extracted nucleic acids are then amplified using either universal primers or species-specific genes before sequencing. Obtained sequences are then compared with sequences available in data bases such as the National Centre for Biotechnology Information - NCBI GenBank to establish the phylogenetic relationship of the isolates (Mayo et al., 2014).

Culture-independent methods that have been used to study seafood spoilage bacteria or seafood borne pathogens include amplified fragment length polymorphism – AFLP (Laursen et al., 2005), denaturing gradient gel electrophoresis – DGGE, repetitive extragenic palindromic – polymerase chain reaction (REP-PCR) (Broekaert et al., 2011), pulsed field gel electrophoresis – PFGE (Fendri et al., 2013), loop-mediated isothermal amplification – LAMP (Han and Ge, 2010), enterobacterial intragenic consensus sequence - ERIC PCR (Kumar et al., 2009), random amplified polymorphic DNA (RAPD-PCR), temporal temperature gradient gel electrophoresis - TTGE, automated ribosomal intergenic spacer analysis (ARISA), real time PCR/quantitative PCR (Zhu et al., 2012), and terminal restriction fragment length polymorphism – TRFLP (Tanaka et al., 2010). Lee and Levin (2010) used real-time PCR to quantify the total viable bacteria present in fish fillets by amplifying a highly conserved bacterial 16S rRNA sequence in conjunction with ethidium bromide monoazide - (EMA) dye to differentiate viable and dead bacterial cells. Although the use of real-time PCR is fast and requires no post PCR steps it cannot differentiate between DNA from viable and dead microorganisms (Gonzalez et al., 1999; Lee and Levin, 2010) . Zhao et al. (2016), used PCR-DGGE to assess the microbial diversity present in shrimp while Bekaert et al. (2015) used a

similar method to characterize *Acinetobacter* spp. *Vibrio* spp. and *Aeromonas* as dominant bacteria in lobster held under different storage conditions. They identified *Psychrobacter* spp. and *Pseudomonas* spp. as dominant spoilage bacteria. Likewise, Svanevik and Lunestad (2011) characterized the microbiota of Atlantic mackerel using a similar method. They identified *Shewanella* spp. *Photobacterium* spp. *Psychrobacter* spp. and *Pseudoalteromonas* spp. as the dominant spoilage bacteria in mackerel.

Over the years, limitations of the culture-independent methods such as the inability to detect the overall microbial community in seafood, high cost, time-consuming and an inability to differentiate the DNA from viable and dead microbial cells, have led to the use of metagenomic methods such as next - generation sequencing (NGS). This is a high-throughput pyrosequencing technology, which is faster and more cost effective in analysing microbial communities in seafood than other methods such as PCR – DGGE (Kim et al., 2014). NGS enables accurate detection and identification of both cultivable and uncultivable bacteria present in low numbers (Ercolini, 2013; Mayo et al., 2014).

#### 1.2.2.2 Spoilage volatile organic compounds (VOC) in seafood

Volatile organic compounds (VOC) are metabolic by-products released by microorganisms during growth or survival in seafood and can be from enzymatic activities within a seafood matrix (Kuuliala et al., 2018). In the context of this review, VOC are metabolites from bacteria or seafood produced during spoilage. The release of VOC in seafood depends on storage conditions (storage temperature and storage period), processing procedure, the type of

spoilage bacteria, the type of seafood and method of detection. VOC indicating seafood spoilage include alcohols, aldehydes, fatty acids, esters, ketones and sulphur compounds such as hydrogen sulphide ($H_2S$), dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide (Kuuliala et al., 2018; Parlapani et al., 2018).

VOC have been investigated to unveil changes in freshness of seafood such as such as oyster (Cruz-Romero et al., 2008), prawns, crabs, clams (Zhang et al., 2010), shrimps (Chinivasagam et al., 1998), salmon (Miks-Krajnik et al., 2016; Wierda et al., 2006), mussels (Aru et al., 2016b) and processed seafood (Chen et al., 2016). Other studies have shown that VOC are also produced by spoilage bacteria in either raw, packaged or processed seafood. Spoilage bacteria genera such as *Shewanella, Pseudomonas, Photobacterium, Brochothrix, Psychrobacter, Pseudoalteromonas* and *Carnobacterium* have been investigated for VOC production in raw, packaged or processed seafood (Broekaert et al., 2013; Jaffres et al., 2011; Kuuliala et al., 2018; Parlapani and Boziaris, 2016; Parlapani et al., 2018) as summarized in Table 1.2, while sensory descriptions of spoilage microbiota are summarized in Table 1.3.

**Table 1.2:** Examples of volatile organic compounds (VOC) produced by seafood spoilage bacteria

| VOC | Seafood | Spoilage bacteria | Storage conditions | Methods | References |
|---|---|---|---|---|---|
| Ethanol, 3-Pentanol<br>3-Methyl-1-butanol<br>2-Methyl-1-butanol<br>2-Ethyl-1-hexanol<br>Heptyl alcohol<br>1-Penten-3-ol | Gutted sea bream<br><br>Packed shrimp | *Pseudomonas* spp.,<br>*Pseudomonas fluorescen,*<br>*Carnobacterium* spp. | Air at 2˚C for 13 days<br><br>MAP at 5˚C for 10 days | SPME-GC/MS<br><br>GC-MS/HPLC | Parlapani et al. (2014)<br><br>Laursen et al. (2006) |
| 2-Penten-1-ol, 1-pentanol,<br>1-hexanol<br>1-decanol, 1-dodecanol | Gutted European sea bass | *Brochothrix thermosphacta*<br><br>*Pseudoalteromonas* spp., | Air at 2˚C for 13 days<br>MAP at 2˚C for 15 days | SPME-GC/MS | Laursen et al. (2006) |
| 2,3-Dimethyl-oxirane<br>2-Butanone, 2-<br>Formylhistamine | Cooked brown shrimp | *Psychrobacter* spp.<br><br>*Shewanella liquefaciens,* | Refrigerated bottles at 4˚C for 9 days | GC–MS/ SIFT-MS | Broekaert et al. (2013) |
| 1-propanol<br>Cyclopentanol<br>2,3-butanediol<br>Isoamylalcohol | Cooked and peeled tropical shrimps | *Carnobacterium maltaromaticum* | MAP at 8˚C | SPME–GC–MS | Jaffres et al. (2011) |

**Table 1.3:** Sensory description of most dominant seafood spoilage bacteria

| Seafood spoilage bacteria | Sensory descriptors | References | Seafood matrix |
|---|---|---|---|
| *Aeromonas salmonicida* | Sour | Mace et al. (2014) | Cooked tropical shrimp |
| *Aeromonas* spp. | Amine, socks, floorcloth | Joffraud et al. (2001) | Cold-smoked salmon |
| *Brochothrix thermosphacta* | Blue-cheese, sour, pungent, | Leroi et al. (1998) | Cold-smoked salmon |
| *Brochothrix* spp. | Butyric acid | Leroi et al. (1998) | Cold-smoked salmon |
| *Chryseobacterium piscicola* | Butter, caramel, sour | Joffraud et al. (2001) | Cold-smoked salmon |
| *Carnobacterium maltaromaticum* | Cheese, feet, sour, fermented, butter | Jaffres et al. (2011) | Cooked shrimp |
| *Hafnia alvei* | Pyrrolidine | Mace et al. (2013b) | Salmon |
| *Serratia liquefaciens-like* | Cabbage, garlic, amine, urine | Jaffres et al. (2011) | Cooked shrimp |
| *Shewanella putrefaciens* | Putrid, $H_2S$ | Leroi et al. (1998) | Cold-smoked salmon |
| *Photobacterium phosphoreum* | Amine, sour | Mace et al. (2014) | Salmon |
| *Pseudomonas fragi* | Fruity, sulphide | Chinivasagam et al. (1998) | Sterile banana prawn |

#### 1.2.2.3 Sensory evaluation (the quality index method)

Sensory evaluation can be used in monitoring the changes in sensory attributes of seafood during storage conditions (Fuentes-Amaya et al., 2016). It is a rapid, but subjective method of seafood quality assessment (Hyldig and Green-Petersen, 2005). The use of sensory methods for quality assessment of seafood requires trained assessors, in some cases involves ethical approval and could be expensive (Fuentes-Amaya et al., 2016). The quality index method (QIM) is a sensory evaluation tool that requires trained assessors and is based on scoring attributes (odour, texture, and appearance) of ice-stored (0°C) seafood that can change during the storage period (Bremner, 1985).The scores obtained can then be used to predict the shelf-life of the seafood based on its storage temperature (for example, Diler and Genç (2018) developed a QIM for rainbow trout). QIM was developed in 1985 by Bremner at the Tasmania Food Research Unit (TFRU), Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia, for the assessment of the freshness of fish (Bremner, 1985). However, it has been used for the assessment of freshness of other seafood such as shucked oysters (He et al., 2002), spiny lobster (Goncalves et al., 2015), and fish (Bonilla et al., 2007; Ritter et al., 2016). QIM has been described as being reliable because it can measure the rate and degree of change in freshness (Goncalves et al., 2015). To avoid undue repetition, more details of QIM are provided in Chapter 6.

## 1.3 Mussels

Mussels, like other seafood, are very nutritious and are the second largest aquaculture product produced worldwide after finfish (FAO, 2009) (Figure 1.3).

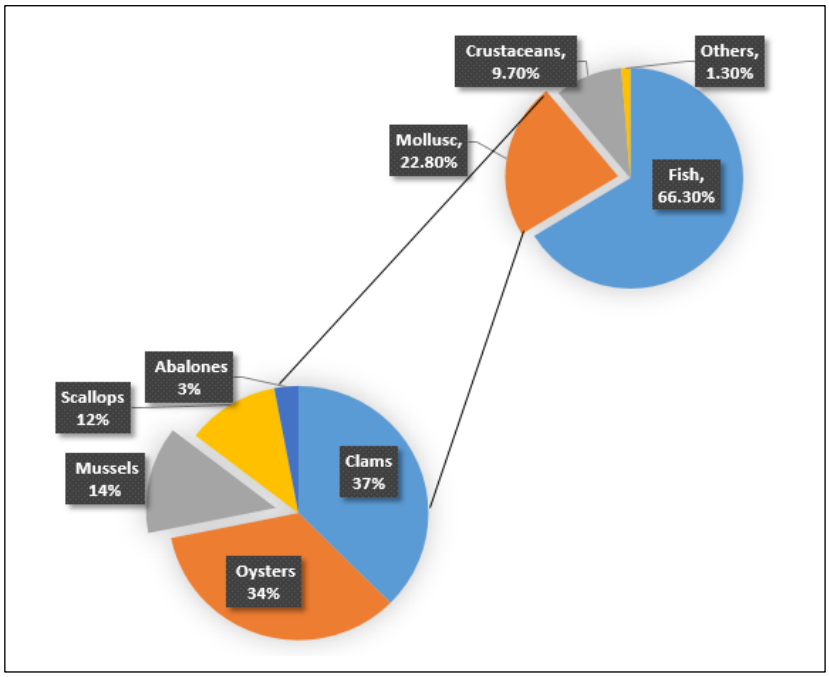


**Figure 1.3:** Global production of seafood (FAO, 2013, 2014).

The two most recognised mussel genera are *Mytilus* and *Perna*. According to Coustau et al. (1991), based on allozyme polymorphism and geographical distributions, the genus *Mytilus* is taxonomically classified into closely related species: *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus trossulus*. These species are known as blue mussel *(M. edulis*), Mediterranean mussel (*M. galloprovincialis*) and Baltic mussel (*M. trossulus* ) in different parts of the world (Gosling, 1992; Koehn, 1991). *M. edulis* is mostly found in temperate cold-water areas of Europe and North America (Innes and Bates, 1999; Lopez et al., 2002). *M.*

*galloprovincialis*, a cosmopolitan species, is commonly found and harvested in the Mediterranean, the Marmara Sea and Bosphorus countries (Erkan, 2005; Ulusoy and Ozden, 2011) and in Australia (Rochfort et al., 2013). The genus *Perna* also has three most-recognised species: *Perna canaliculus* also known as the green lipped New Zealand mussel, *Perna viridis* and *Perna perna* (Dias et al., 2013).

### 1.3.1 Mussel aquaculture

Globally, Europe is still the largest producers of mussels. Spain is the highest producer of blue mussels and followed by Italy (Rochfort et al., 2013). However, due to the high demand for blue mussels in Europe, Belgium, France and Italy are the highest importers of mussels. China is another major consumer of blue mussels while the Netherlands, New Zealand and Spain are the largest exporters of mussels (Bennison, 2002).

### 1.3.2 Mussel farming in Australia

In Australia, *M. galloprovincialis* is the most widely distributed mussel and is often referred to as the Australian blue mussel – registered through the Australian Fish Names Committee and listed in the Australian Seafood Handbook (Bennison, 2002). All Australian blue mussels are harvested in accordance with the Australian Shellfish Quality Assurance Program and can be traced from farm to plate (Bennison, 2002).

Mobsby and Koudah (2017) reported that over 3600 tonnes (t) of mussels were harvested by Australian producers between 2015 – 2016 and valued at $10.7M: Western Australia (198t), $0.8M; South Australia (2,088t), $4.4M; Victoria (764t), $3.2M and Tasmania (575t), $2.3M. There are various mussel farmers in Australia among which are: Kinkawooka, Boston Bay Mussels, Spring Bay Seafood (uses a modified atmosphere packaging method to sell live mussels) and Spencer Gulf Mussels (http://seafoodfrontier.com.au/product/mussels/) - sells live mussels in vacuum-packed containers. Mussel farmers in South Australia have also been selling live mussels using modified atmosphere packaging method.

### 1.3.3 Habitat, biology and distribution of mussel (*Mytilus)*

The life cycle of mussels involves four stages, namely spawning, trochophore larvae, veliger larvae and adult (Figure 1.4). Although it is hard to determine the gender of mussels from the shell when cooked, female gonads are orange coloured while male gonads are whitish (Figure 1.5). Australian blue mussels are found at low tide level down to 10 metres depth and they reach a maximum growth of 40 mm within 4 to 6 months (PIRSA, 2018).

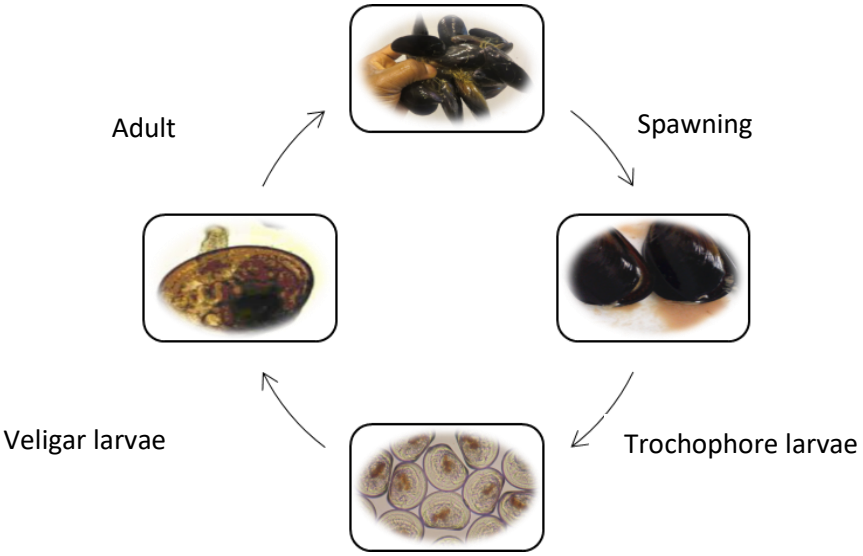


**Figure 1.4:** Life cycle of mussels (Goulletquer, 2004).

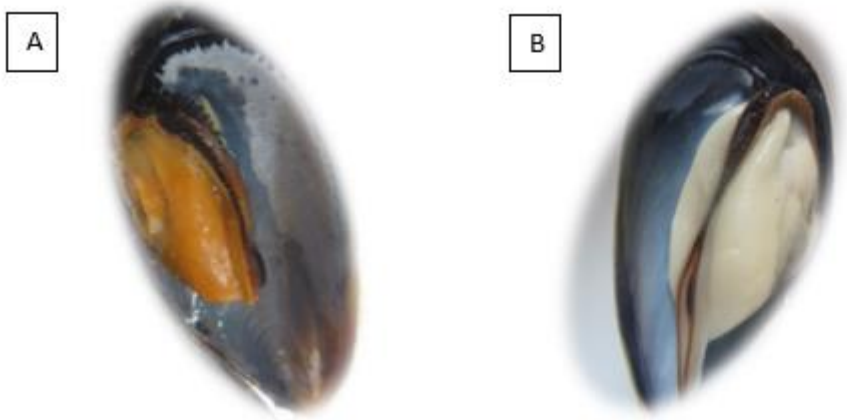


**Figure 1.5:** Female – orange (A) and male – cream (B) blue mussels.

### 1.3.4 Mussel culture methods

Mussels can be cultured in the following three major ways: suspended, bottom and bouchot culture methods (Lauzon-Guay et al., 2005). Suspended culture uses rafts or longlines; bottom culture involves seeding intertidal beds while suspended culture has advantages of faster growth rates and higher tissue-to-shell ratio over bottom culture (Simeona, 1990).

Mussel culturing in Australia is mostly done using floating longlines as practised in Canada some parts of Europe like Spain, France, Germany, Netherlands and Sweden while Ireland, United Kingdom and Norway practise bottom culture (Mallet and Myrand, 1995; Smaal, 2002). However, a major problem with bottom culture is predation of mussel spat (seed) by crustaceans like crabs and starfish (Smaal, 2002).

Bouchot cultures involve the use of wooden poles that are perpendicularly placed to the shore and stuck in the sandy mud within the gently sloping intertidal strip (Lauzon-Guay et al., 2005). Mussels are transferred either as seed attached on collecting ropes or as juveniles, wound up around the 2 m poles and allowed to grow to marketable size within 2 years (Garen et al., 2004). Raft systems are easier to work with in rough weather and occupy fewer surfaces compared to the longline culture method (Karayucel and Karayucel, 1999).

### 1.3.5 Post-harvest handling of mussels

After harvest, cultured mussels are sent to a primary processing plant for washing and declumping before being debyssed, packaged and marketed (Harding et al., 2004). Harvested mussels must be properly processed to reduce the microbial load of pathogenic and spoilage bacteria. Poor handling conditions can have a negative impact on the mussels and may induce stress responses which can lead to reduced quality and shelf- life because the health of mussels, efficiency of depuration practice, post-harvest handling can all affect the shelf-life (Bernardez and Pastoriza, 2013).

During processing, mussels can be contaminated with *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes and Clostridium perfringens* from handlers and or processing equipment (Graczyk and Schwab, 2000; Rippey, 1994). For example, Misrachi et al. (1991) reported the presence of *Listeria* spp. in smoked mussels consumed in Tasmania, Australia while Brett et al. (1998) reported two cases of listeriosis caused by *Listeria monocytogenes* due to consumption of smoked mussels (*Perna canaliculus*) in Auckland, New Zealand. The presence of *Listeria* in smoked mussels could be contamination from the processing plants. Biofilm-forming *L. monocytogenes* have been reported in raw mussel processed products and three processing plants in New Zealand (Cruz and Fletcher, 2011) and are related to reported human cases (Cruz et al., 2014).

### 1.3.6 Mussel products

Globally, mussels are mostly sold as unprocessed and processed products in jute bags or hermetic packages (Bernardez and Pastoriza, 2013). Among the raw products are shucked mussels (Goulas et al., 2005), live mussels (Bernardez and Pastoriza, 2011, 2013) while smoked, canned (Sengor and Kalafatoglu, 2004; Turan et al., 2008) and pickled mussels (Goulas and Kontominas, 2005; Ulusoy and Ozden, 2011) are processed products. More recently, due to the nutritional quality of mussels (Bongiorno et al., 2015), *Sous vide* cook-chill mussel have also been marketed (Bongiorno et al., 2018). Goulas et al. (2005) stated that consumers prefer live mussels because of the nutritional qualities and organoleptic properties. The use of hermetic containers for marketing live mussels was developed by Spanish researchers, but the patenting of the hermetic container method might have affected the liberal use of hermetic containers for live mussels (Pastoriza et al., 2005). In a study by Pastoriza et al. (2004), it was observed that tightly packed live mussels in hermetic containers improved shelf-life from 2-3 days to 6 days since they could not gape.

Australian blue mussels are usually marketed as fresh live mussels in vacuum or modified atmosphere packaging (MAP). While countries like Canada, Chile, Asian countries (China, Japan, Thailand, Philippines and European countries sell mussels as shucked mussel (frozen mussel meat), smoked (meat), marinated (whole or half shelled), bottled or canned (preserved in edible oil or vinegar).

Value-added products of mussels are usually prepared with preservatives added to prolong the shelf-life. However, preservative may injure live mussels thus cannot be used. Various

studies have been carried out on the shelf-life of live and shucked mussels (Bernardez and Pastoriza, 2011, 2013; Goulas et al., 2005; Pastoriza et al., 2004). Mussel size and headspace oxygen have been found to affect the shelf-life of live mussels. An 8-day shelf-life of live mussels packed with 75 – 85% $O_2$ at 2 ± 1 °C storage temperature was reported by Bernardez and Pastoriza (2011) using the following parameters: mortality, microbiological and chemical analysis and organoleptic assessment. A similar result of 8 – 9 days shelf-life of live mussels packed with 83% $O_2$ and stored at 2 °C was obtained in another study by the authors (Bernardez and Pastoriza, 2013).

Based on sensory evaluation and chemical analysis, Erkan (2005) reported that the shelf-life of shucked mussels stored at 4°C was 4 days. However, a change of headspace gas composition has been used to extend the shelf-life of shucked mussels. Goulas et al. (2005) reported 11 – 12 days shelf-life of shucked mussels packed with 40% $CO_2$, 30% $O_2$ and 30% $N_2$ stored at 4°C. showed that the shelf-life of wild shucked mussels was 8 days when packed with either 80% $CO_2$/20% $N_2$ or 65% $CO_2$ /35% $N_2$ when stored at 2 ± 1°C. Results by Aru et al. (2016b) showed that shucked mussels (*M. galloprovincialis*) had 10 and 6 days shelf-life when stored at 0 °C and 4 °C, based on the microbiological analysis only. Aru et al. (2016b) reported that storage at low temperature (0 – 6°C) helps in retaining the quality and organoleptic attributes of seafood. Higher temperature (> 7°C) increases both metabolic activities of mussels and microbial proliferation thereby leading to quality loss and shelf-life limitation (Bernardez and Pastoriza, 2011, 2013).

### 1.3.7 Modified atmosphere packaging and shelf-life extension of mussels

In the past two decades, modified atmosphere packaging (MAP), has become a increasingly popular preservation technique in the food industry due to the changes in storage, product distribution, and marketing of raw and processed food products (Ozogul et al., 2004). MAP is a hurdle technology involving deliberate modification of gas composition around food such as seafood products to extend the shelf-life in combination with low storage temperature (Pastoriza and Bernárdez, 2012). The gases compositions usually used either singly or in combination in MAP are oxygen ($O_2$), carbon dioxide ($CO_2$) and nitrogen ($N_2$) (Davies, 2003; Genigeorgis, 1985). $CO_2$ has a bacteriostatic effect and selectively inhibits the growth of aerobic bacteria, yeast and mould by prolonging the lag phase (Genigeorgis, 1985; Reddy et al., 1991). It has been used to preserve the quality and extend the shelf-life of seafood such as fish (Alfaro and Hernandez, 2013), shucked mussels (Goulas et al., 2005) and half shell oysters (Chen et al., 2017). $O_2$ inhibit the growth of anaerobic spoilage bacteria while $N_2$ is used in combination with either or both $CO_2$ and $O_2$ to prevent the packs collapsing (Genigeorgis, 1985; Pastoriza et al., 2004).

The post-harvest conditions of raw seafood, the packaging materials, the temperature and the gas mixtures can influence the shelf-life of seafood products (Farber, 1991). According to Caglak et al. (2008), the guiding principle behind the effectiveness of MAP in extending the shelf-life of food products is the inhibition of both microbial growth and oxidizing reactions. Diverse studies investigating the effectiveness of MAP in extending shelf-life of mussel products have been conducted (Table 1.4).

Goulas et al. (2005), investigated microbiological and sensory changes of shucked mussel (*M. galloprovincialis*) stored under MAP at 4$^{o}$C. There was variation in the shelf-life of the product based on packaging conditions. MAP with a gas composition of 80% $CO_2$ and 20% $N_2$ extended the shelf-life to 14 – 15 days compared to 11 – 12 days for MAP with 40-50% $CO_2$, 30 - 50% $N_2$ and 0-30% $O_2$ and 5 – 7 days shelf-life for raw mussels under the same conditions of storage. In a shelf-life study by Madigan (2014), it was reported that MAP (30% $CO_2$ and 70% $N_2$) slowed the growth of spoilage bacteria in half shell Pacific oysters stored at 4°C for 9 days thereby extending the shelf-life. Another study by Masniyom et al. (2011) revealed that MAP (80% $CO_2$, 10% $O_2$ and 10% $N_2$) gave 12 days shelf-life for green mussel (*Perna viridis*) and it was concluded in this study that oxygen should be included in MAP to avoid growth of anaerobic bacteria. Bernardez and Pastoriza (2011) studied the efficacy of MAP gas mixtures with low and high oxygen in comparison with the size of mussel used (30 and 44 mussels/kg). This study showed that higher oxygen levels (75 – 80%) in the gas mixture, with fewer mussels and a storage temperature of 2 ± 1$^{o}$C favoured stability of packaged live mussel and with fewer metabolic products.

The different shelf-lives reported in the studies could be due to the levels of $CO_2$ and $O_2$ used. Higher $CO_2$ resulted in increased inhibition of aerobic bacteria. Similarly, higher $O_2$ resulted in increased inhibition of anaerobic bacteria. Variability in the results could therefore be due to increased anaerobic and aerobic bacteria depending on the gas composition used. Similarly, molluscs like mussels are likely to have highly variable numbers of bacteria in their normal microbiota (Utermann et al., 2018). Therefore, mussels with fewer initial bacteria in a pack may perform better others. The method of cooking and the

criteria used for sensory evaluation could affect the results. For example, in the study of Bernardez and Pastoriza (2011), mussels used for sensory evaluation were steam cooked for 4-6 minutes at 100°C, while Goulas and Kontominas (2005) reported cooking mussels for 2 minutes in a microwave. The overheating of mussels during cooking could affect the texture thereby resulting false negative rejection of the mussels by the panellist earlier than expected. Ovissipour et al. (2013) reported that heating mussels (*Mytilus edulis*) for 5 minutes at 70 – 90°C resulted in shrinkage, protein denaturation and texture loss.

**Table 1.4:** Summaries of studies on the effect of modified atmosphere packaging (MAP) on the shelf-life of mussel products

| Mussel products | Microbial analysis | Storage temperature | Days of storage | MAP conditions | Major findings | References |
|---|---|---|---|---|---|---|
| Shucked mussel (*Mytilus galloprovincialis*) | Total viable count, *Pseudomonas* spp., lactic acid bacteria and $H_2S$-producing bacteria. | 4°C | 0 – 15 days | 50%$CO_2$+50%$N_2$<br>80%$CO_2$+20%$N_2$<br>40%$CO_2$+30%$N_2$+30%$O_2$ | 50%$CO_2$+50%$N_2$ and 40%$CO_2$+30% $N_2$+30%$O_2$ extended the shell life of the product by 11 – 12 days. | Goulas et al. (2005) |
| Shucked mussels | Total viable, lactic acid bacteria and psychotropic bacteria counts | 2 ±1 °C | 0 – 12 days | 50%$CO_2$+50%$N_2$<br>80%$CO_2$+20%$N_2$<br>65%$CO_2$+35%$N_2$ | Gas composition of 80%$CO_2$+20% $N_2$ was must effective. It extended the shell life by 14 – 15 days compared to 5 – 7 days shell life of raw mussel. | Caglak et al. (2008) |
| Stuffed mussels (*M. galloprovincialis*) | Total viable aerobic bacterial count, psychrophilic count and total anaerobic bacteria | 4°C | 1-15 days | 50%$CO_2$+50%$N_2$<br>100% $CO_2$ | Gas composition of 50%$CO_2$+50% $N_2$ more effective in extending the shell life of mussel by 11 days. | Ulusoy and Ozden (2011) |
| Green mussel (*Perna viridis*) | Total viable count, lactic acid bacteria | 4 °C | 15 days | 40%$CO_2$+10%$O_2$+50%$N_2$<br>60%$CO_2$+10%$O_2$+30%$N_2$<br>80%$CO_2$+10%$O_2$+10%$N_2$<br>100%$CO_2$ | 80%$CO_2$+10%$O_2$+10%$N_2$ shell life extended shell life of green mussel by 12 days. It was concluded in this study that oxygen should be included in MAP packaging to avoid growth of anaerobic toxigenic bacteria | Masniyom et al. (2011) |

## 1.4 Research gaps

Although mussels are depurated before marketing, the best depuration practice is not fully known. Incomplete emptying of the gut during depuration could lead to contamination of pouch water, which provides a suitable medium for microbial growth. The duration of depuration could therefore play significant role in quality and spoilage of mussels. Aside from appropriate temperature, live mussels require oxygen for survival throughout the supply chain. Optimised storage temperature, depuration and initial oxygen are therefore required to retain the highest quality of live mussels for the longest time during the supply chain. There is little information on how microbial communities and volatile organic compounds (VOC) change in live mussels across their storage period. No study has linked changes in microbial communities with VOC to describe the mechanism of spoilage.

## 1.5 Research hypotheses

1.5.1 Lack of efficient depuration prior to packaging contributes biological burden and microbial growth leading to objectionable smell in the sealed packs.

1.5.2 The microbial communities in packaged mussels determine the metabolites and off-odours produced.

1.5.3 Sensory and quality assessment of live mussels can be determined using a subjective evaluation (quality index method - QIM).

## 1.6 Research objective

The overall objective of this study is to understand the process of microbial spoilage in MAP live mussels to optimise processing that can lengthen the shelf-life.

## 1.7 Research aims

1.7.1 To optimise post-harvest storage conditions of freshly harvested live mussels.

1.7.2 To evaluate freshness and spoilage VOC of MAP live mussels.

1.7.3 To investigate the microbial community and its succession in MAP live mussels.

1.7.4 To assess spoilage potential and production of VOC by hydrogen sulphide-producing bacteria isolated from live mussels.

1.7.5 To develop a subjective method (quality index method) for sensory quality and shelf-life assessment of MAP live mussels.

## 1.8 Thesis structure

There are seven Chapters in this thesis comprising of introduction, five experimental Chapters and a general discussion of the project. Each experimental Chapter stands alone for publication purposes but is linked with others. However, there is unavoidable repetition of some of the methods used in the experimental Chapters.

Chapter 1 gives a general introduction to seafood spoilage; microbial spoilage; current methods of assessment of spoilage in seafood that involves the use of culture-dependent and culture-independent methods; sensory evaluation and production of volatile organic compounds. It also discusses mussel aquaculture, types of mussel products, mussel farming in Australia with a view to identifying the knowledge gaps that will form the purpose of the study.

Chapter 2 describes the optimisation of duration of depuration, of storage temperature and of headspace oxygen on the microbial quality and sensory quality of live mussels stored at 4°C.

Chapter 3 describes the evaluation of freshness and spoilage VOC in MAP live mussels during storage at 4°C using headspace-solid phase micro-extraction gas chromatography–mass spectrometry (HS-SPME/GC-MS).

Chapter 4 describes the diversity of spoilage bacteria and their succession in MAP live mussels at storage temperature. High-throughput pyrosequencing was used to identify taxa present initially and with storage under MAP at 4°C.

Chapter 5 assesses the spoilage potential and VOC of hydrogen sulphide - producing bacteria isolated from modified atmosphere packaged live mussels. One of the isolates (*Shewanella baltica*) was then used to inoculate sterile mussel broth stored for 10 days' storage at 4°C and the VOC identified. These were then compared with VOC in the MAP mussels.

Chapter 6 describes the development and evaluation of a quality index method for evaluation of sensory quality and shelf-life of MAP live mussels using sensory assessors. The model developed predicted the shelf-life of live mussels packed at optimised gas composition, duration of depuration and storage temperature.

Chapter 7 synthesises the results of all the experimental Chapters to describe spoilage mechanisms. It also describes the limitations of this study and areas of future work.

# Chapter 2: Optimisation of post-harvest storage conditions of freshly harvested live mussels for modified atmosphere packaging

## 2.1 Introduction

Mussels are the third most widely produced bivalve shellfish in the world aside from oysters and scallops and the demand and consumption of mussels continue to increase (FAO, 2017). Mussels are nutritious, rich in essential minerals and vitamins required in healthy human diet (Goulas et al., 2005). Mussels are also rich in free amino acids and high polyunsaturated fatty acids (Fernandez et al., 2015; Goulas et al., 2005).

Mussels are mostly sold as processed and unprocessed products in hermetic packages - plastic pouches (Bernardez and Pastoriza, 2013; Goulas et al., 2005). The unprocessed products are live, shucked, unshelled and stuffed mussels while processed products are smoked and pickled (Goulas et al., 2005; Ulusoy and Ozden, 2011). However, due to nutritional qualities and organoleptic properties, fresh live mussels are being preferred to other mussel products by consumers (Goulas et al., 2005). Technological advancement and improved supply chain logistics that helped sales of live mussels have resulted in increased shelf-life, increased demand and huge financial benefits to mussels' farmers (Bernardez and Pastoriza, 2013; Goulas et al., 2005).

Current industrial practice indicates shelf-life of live mussels in modified atmosphere packaging (MAP) is limited to 7-10 days due to either or both autolytic degradation of mussels and bacterial growth (Bernardez and Pastoriza, 2011, 2013; Pastoriza et al., 2004). The result is an off-smell that renders the product unacceptable and decomposition of mussels. At the time of industrial packaging, mussel condition is influenced by the health of mussels at

harvest, the method of harvest, the efficacy of depuration prior to processing and packing, handling of mussels during harvest, post-harvest period and the holding temperature. These factors contribute to stress in live mussels that could limit marketability shelf-life due to increased mortalities and spoilage thereby resulting in economic loss to the producers and retailers (Anacleto et al., 2013; Bernardez and Pastoriza, 2013).

During depuration, also known as wet storage, freshly harvested mussels are placed in static filtered seawater for some hours to purge the mussels and empty their guts. Although most of the mussels empty their guts, this still leaves a small number of animals that could subsequently contaminate pouch water used in the packs. Faeces provides both nutrients and inoculum for bacterial growth. It is therefore important to establish the best duration of depuration in hours that could be effective. Depuration could impact mussel quality, meat condition index, mortality and responses of mussels to stress (Gallardi et al., 2014).

Low temperatures (0 – 6°C) are required for extending the shell-life of seafood (Aru et al., 2016b). Low storage temperature at 2°C to 3°C helps in preservation of organoleptic properties of live mussels while storage at high temperature (7°C) alters organoleptic properties, increases mortality and increases metabolic activities (Bernardez and Pastoriza, 2011, 2013). Live mussels require oxygen for survival. Studies on the effects of headspace oxygen concentration and gas mixtures on quality and shelf-life of live mussels have been reported (Bernardez and Pastoriza, 2011, 2013; Pastoriza et al., 2004). Three studies by the same research group have reported effect of storage temperatures on live mussels. The first study in 2004 reported that storage of live mussels packed with 75% $O_2$ and stored at 2 ± 1°C

improved survival compared to low oxygen packed mussels (Pastoriza et al., 2004). The second study reported stability of live mussels stored at 2 – 3°C (Bernardez and Pastoriza, 2011) while third study reported that the quality of live mussels packed with 83% $O_2$ and stored at 2°C remained maintained while storage at 7°C impairs viability of live mussels (Bernardez and Pastoriza, 2013).

Some pertinent questions remained unanswered from the scientific literature: (1) What is the optimum headspace oxygen for the extension of the shelf-life of live mussel? (2) What is the optimum depuration treatment prior to packing mussels? (3) What is the optimum post-packaging storage temperature? (4) What is the spoilage mechanism of live mussels? This study therefore aims to optimise depuration time, headspace oxygen and storage temperature of freshly harvested live mussels and to identify the mechanism of spoilage relevant to mussels harvested from Tasmanian/Southern Australian growing areas and their use in MAP.

## 2.2. Materials and methods

To achieve the aim of this research, the study was conducted in three phases.

### 2.2.1 Phase 1: Optimization of storage temperature

#### 2.2.1.1 Mussels at harvest

Live mussels used were of standard commercial size (average weight: 21.91 ± 0.52 g; average length: 8.25 ± 0.61 cm and average width: 3.73 ± 0.25 cm) with slightly brittle shells were harvested from 10 – 15 m deep at Spring Bay Seafoods farm located at Triabunna, east coast Tasmania, Australia (Lat: 42˚ 32.2021', Long: 147˚58.7809'). Mussels were then cleaned while byssus threads were mechanically removed. Thereafter, mussels were graded and stored in aerated static tank overnight at 4°C. Water temperature at harvest was 13.1 ± 0.14˚C. Data loggers were used to monitor temperature variation during transportation. Samples were transported to the Aquaculture Centre at the University of Tasmania, Launceston, Australia in two ice-packed Styrofoam boxes (≈ 4˚C). On arrival at the Aquaculture Centre (≈ 6 – 8 hrs post-harvest), mussels were stored overnight in a 50 L static tank at 4˚C. Thereafter, mussels were transferred to 250 L recirculating aquaculture systems (RAS) with 5 – 10% volume of water changed once a day and acclimatised for 1 week. The water quality (total ammonia nitrogen, pH, salinity, nitrate and nitrite, temperature, dissolved oxygen concentration, saturation) and mortality were monitored daily.

#### 2.2.1.2 Modified atmosphere packaging (MAP) of live mussels

Six to eight mussels (161 ± 1.41 g) with 100 mL of sterile town supply freshwater (added to mimic commercial practice) were packed in 9 x 7 x 40 cm (BT97/40) Barrier black trays (Alto

packaging Ltd, Australia). Trays with mussels were transferred to a semi-automatic MAP machine (Multivac T100, Germany). The machine was equipped with a vacuum pump for the evacuation of air (3 mbar) before filling with 100% pre-set headspace oxygen gas composition (Food grade oxygen, BOC, Australia). The oxygen transmission rate (OTR) for the cover film was 22 cc/$m^2$/day/atm 23°C 0% relative humidity (RH) and the water vapour transmission rate (VPR) was 22g/$m^2$/day/ at 37°C and 90% RH. Sealing was carried out at 140 - 142˚C. The final pressure in each pack was 1 mbar. All sealed samples were stored at 4°C for 10 days post packing.

#### 2.2.1.3 Storage of MAP live mussels.

A total of 65 packs of MAP live mussels were stored at the following temperatures: 4˚C, 6.5˚C, 10˚C and 13˚C. Low temperature (4 – 6.5°C) was to mimic commercial practice while 10 and 13°C were chosen to mimic temperature abuse during handling prior to consumption. At 0 hr, duplicate samples were analysed. Thereafter, triplicate biological samples were opened and analysed for each temperature and sampling point. Briefly, 15 samples were stored at 13°C and were evaluated at 18 hr, 30 hr, 42 hr, 54 hr and 78 hr (3 days 6 hrs). At 10°C, 12 samples were stored and evaluated at 24 hr, 48 hr, 72 hr and 96 hr (4 days). However, 15 samples were stored at 6.5˚C and evaluated at 24 hr, 48 hr, 72 hr, 96 hr and 120 hr (5 days). Lastly, 15 samples were stored at 4˚C and evaluated at 48 hr, 96 hr, 144 hr, 192 hr and 240 hr (10 days).

#### 2.2.1.4 Headspace analysis of MAP live mussels

Prior to opening the samples, $CO_2$ and oxygen levels in the headspace of all the samples were analysed with a gas analyser machine (PBI Dansensor CheckPoint II, Denmark). A rubber septum was placed on each pack and the needle was inserted after initial calibration with ambient air (Milne and Powell, 2014). Gas levels were reported as percentages (%).

#### 2.2.1.5 Microbiological analysis

Three replicates samples were analysed for total viable bacteria from day 0 – 10. Microbiological analysis was carried out as follows: On each sampling time (day), 70% ethanol was used to disinfect the surface of the packs before opening with sterile scissors. Thereafter, from the total mussel meat shucked (6 – 8 mussels), 15 g of mussel meat was added to 135 mL (ratio 1:10) of sterile alkaline peptone water (pH 8.6 ± 0.2 at 25°C) containing bacteriological peptone 10 g (LP003T, Oxoid) and 20 g NaCl (Code 0314B92, Amresco, USA) (Dabade et al., 2015). Standard Plate Count (SPC) agar (LP0011, Oxoid, England) was used for the enumeration of total viable bacteria count. The SPC agar was prepared with sterile distilled water following manufacturer's instruction and autoclaved at 121°C for 15 minutes. The molten sterile agar was allowed to cool to 45°C before pouring into sterile Petri dishes (plates). Samples were homogenised for 60 seconds before serially diluting 1 mL of each sample in sterile 9 mL of sterile saline (0.85% w/v). Thereafter, 10 µL of decimal dilutions from each sample were spread on SPC agar. The plates were incubated for 48 hr at 25°C. Colonies on each plate were counted as colony - forming units (CFU) and log-transformed to log CFU $g^{-1}$ or log CFU $mL^{-1}$ (Powell et al., 2015). The microbiological data obtained were fitted by Baranyi's model (Baranyi and Roberts, 1994) using the computer program DMFIT from

ComBase (https://www.combase.cc/index.php/en/) to estimate the lag phases at each storage temperature.

#### 2.2.1.6 Physico-chemical analysis

The dissolved oxygen (DO) in air saturation (%) of pouch water was measured immediately after opening of the packs with a DO meter (Orion Star A223, Thermo Scientific). The pH of both pouch water and mussel meat homogenised as described above was analysed using calibrated pH meter (ATI Orion Research Model 250A, USA) at 25°C (Noseda et al., 2012).

### 2.2.2 Phase 2: Optimisation of headspace oxygen

#### 2.2.2.1 Sample source and packaging

Freshly harvested, cleaned, graded and standard commercial size mussels (65 – 80 mm) harvested from the same location as stated in phase 1 (section 2.2.1.1) were supplied by Spring Bay Seafoods. The mussels were not depurated before delivering to the laboratory. They were only harvested, washed, debearded and packed in ice-packed Styrofoam. Samples were in two batches. All samples were harvested very early in the morning and depurated for 4 hrs before dividing to two batches. The first batch was packed under commercial conditions at the factory site. The second batch was transported to the laboratory under the same conditions described in phase 1 (section 2.2.1.1) before packing as described in phase 1 (section 2.2.1.2) with the following gas compositions: 100% $O_2$ (M100), 80% $O_2$ and 20% $N_2$ (M80) and 60% $O_2$ and 40% $N_2$ (M60). Commercially-depurated mussels ( further referred to as commercial packs) packed with 60% $O_2$ was used as reference.

#### 2.2.2.2 Storage of MAP live mussels

Triplicates of all the treatments were stored at 4˚C for 10 days.

#### 2.2.2.3 Headspace analysis of MAP live mussels

Prior to opening the samples, carbon dioxide and oxygen levels in the headspace of all the samples was analysed according to section 2.2.1.4 above.

#### 2.2.2.4 Microbiological analysis

Microbiological analysis was carried out as described in phase 1 (2.2.1.5). However, an objectionable sulphide smell observed on day 7 and 10 of reference and lab-packed samples in phase 1 was also investigated. Hydrogen sulphide ($H_2S$) - producing bacteria were qualitatively isolated from both mussel meat and pouch water with Iron agar (IA) using the following formulations: bacteriological peptone 20 g (Code LP0037, Oxoid, UK), Lab lemco powder 3 g (Code L29, Oxoid, UK), yeast extract 3 g (Code LP0021, Oxoid, UK), ferric citrate 0.3 g (Code 28381, BDH, UK), sodium thiosulphate 0.3 g (Code 517, Ajax Chemicals, Australia), cysteine 0.6 g (Code 37055, BDH, UK), NaCl 5 g (Code 0314B92, Amresco, USA) and agar 15 g (Code LP0011, Oxoid, UK). Ten microliters of each of mussel and pouch water samples was then spread on IA. All plates were incubated for 48 hr at 25°C (Gram et al., 1987; Parlapani et al., 2015).

#### 2.2.2.5 Physico-chemical analysis

Aside from pH measured as described in phase 1 (2.2.1.6), total ammonia nitrogen – TAN (mg $mL^{-1}$) of pouch water of the samples was analysed with a commercial kit (API Mars Fishcare,

North America) following manufacturer's instructions while the tolerance limit was 5 mg $mL^{-1}$ as stated by Barrento et al. (2013). TAN measured was based on the colorimetric scale of 0 ppm (orange) – 8 ppm (deep green) provided by the manufacturer.

#### 2.2.2.6 Meat condition index

The meat condition index (MCI) used as marketability attribute of the mussels was investigated as measures of mussel meat quality. The MCI of each sample was carried out by selecting 5 – 7 mussels from each pack, weighing them and then cooked for 3 minutes in 1500 W microwave oven (Palsonic, Australia). The meat was then removed and weighed (dry meat). The MCI was analysed as (dry meat (g) / total wet weight) x 100 % (Irisarri et al., 2015; Orban et al., 2002). As practised by the commercial supplier of mussels used in this study, a meat condition index of above 30% was used as a measure of marketability meat yield.

### 2.2.3 Phase 3: Optimisation of depuration practices

#### 2.2.3.1 Laboratory depuration

The laboratory depuration system scaled down from the commercial system used on the farm consisted of 10 L open containers, fitted with air line for aeration, a filter to collect mussel faeces and a basement (Figure 2.1). Each tank contained 3 kg of mussels and saltwater (10 L, salinity 35 ppm) obtained from Low Head, Tasmania for depuration. The mussels were not depurated before delivering to the laboratory. The volume of the tank and quantity of mussels was scaled (1:10) to mimic commercial practice (300 kg mussels in 1000 L saltwater). All the tanks were set up concurrently on a platform placed in a temperature (4°C) controlled

room. Depuration was carried out for 4 hr (commercial practice), 8 hr and 12 hr in triplicate (Figure 2.2).

### 2.2.3.2 Modified atmosphere packaging and storage of MAP live mussels

After each depuration period, mussels were transferred in an ice packed Styrofoam box for packaging. The packaging was as described in phase 1 with a headspace of 80% $O_2$ and 20% $N_2$. Triplicate samples of each replicate of the treatment were stored at 4˚C for 15 days. Commercial samples packed from the same batch of harvested mussels were used as a reference.

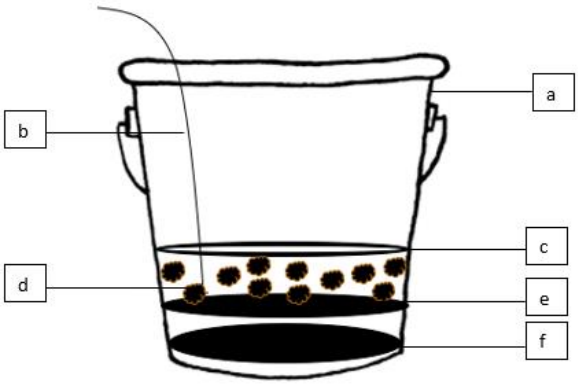


**Figure 2.1:** Laboratory depuration (a) 10 L tank (b) air line for aeration (c) saltwater level (d) standard commercial size mussels – 3kg (e) filter to collect mussel faeces (f) basement.

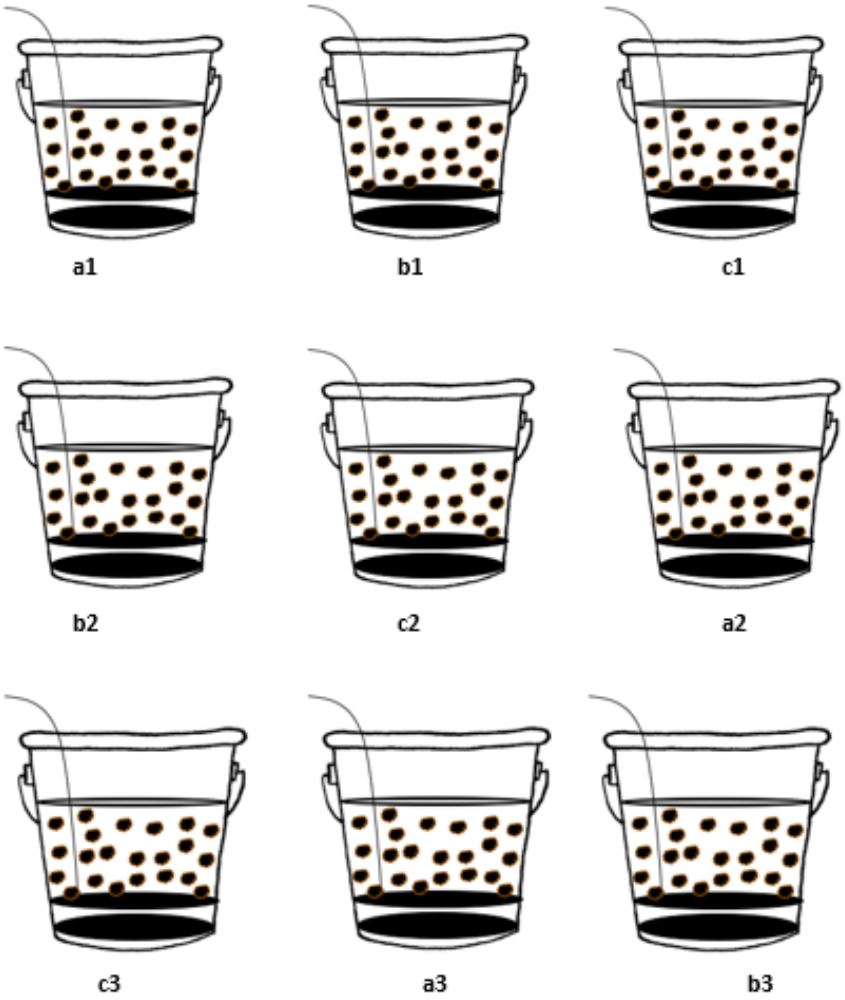


**Figure 2.2:** Laboratory depuration of mussels for (a) 4 hr (b) 8 hr (c) 12 hr. All treatments were in triplicate (a1-3, b1-3 and c1-3).

#### 2.2.3.3 Headspace, microbiological, physico-chemical, meat condition and cook loss analysis of MAP live mussels

The headspace of each pack was analysed for oxygen consumption and production of $CO_2$. Analysis was carried out as outlined in phase 1. Microbial quality and qualitative assessment of spoilage bacteria with focus on hydrogen sulphide-producing bacteria was carried out as stated in phases 1 and 2. The pH of the pouch water and the homogenised mussel meat were analysed as described in phases 1. Meat condition and the total ammonia nitrogen – TAN (mg $mL^{-1}$) were analysed as described in phase 2.

### 2.2.4 Statistical analysis

Microbiological data were expressed as the log of colony-forming units (CFU) per gram or millilitre (log CFU $g^{-1}$ or log CFU $mL^{-1}$) and microbial limit was set at 6 log CFU $g^{-1}$ or $mL^{-1}$ as recommended by (National Advisory Committee on Microbiological Criteria for Foods, 1992). The microbiological data obtained in Phase 1 were fitted by Baranyi's model (Baranyi and Roberts, 1994) using the computer program DMFIT from ComBase (https://www.combase.cc/index.php/en/) to estimate the lag phase at each storage temperature. The significant differences were tested at $p < 0.05$ based on analysis of variance (ANOVA), Tukey's post-hoc multiple comparison tests (Statistical Package for the Social Sciences SPSS version 24 – IBM) and multiple regression (Microsoft Excel 2018).

## 2.3. Results – Phase 1: Optimisation of storage temperature

### 2.3.1 Headspace oxygen of MAP live mussels

Phase 1 of this Chapter aimed to optimise storage temperature required for modified atmosphere packaged (MAP) live mussels in relation to headspace oxygen consumption, dissolved oxygen, pH and microbial quality (total viable count -TVC) during storage at different temperatures. Samples were packed with 100% oxygen and stored for 10 days at the following temperatures: 4°C, 6.5°C, 10°C and 13°C.

The headspace oxygen remained high in all cases above 95% (Figure 2.3). There was a gradual slight decline with storage at 10°C which was significant ($p<0.05$) by 48 hr. In samples stored at 13°C, a significant ($p<0.05$) decline was observed by 30 and 42 hr.

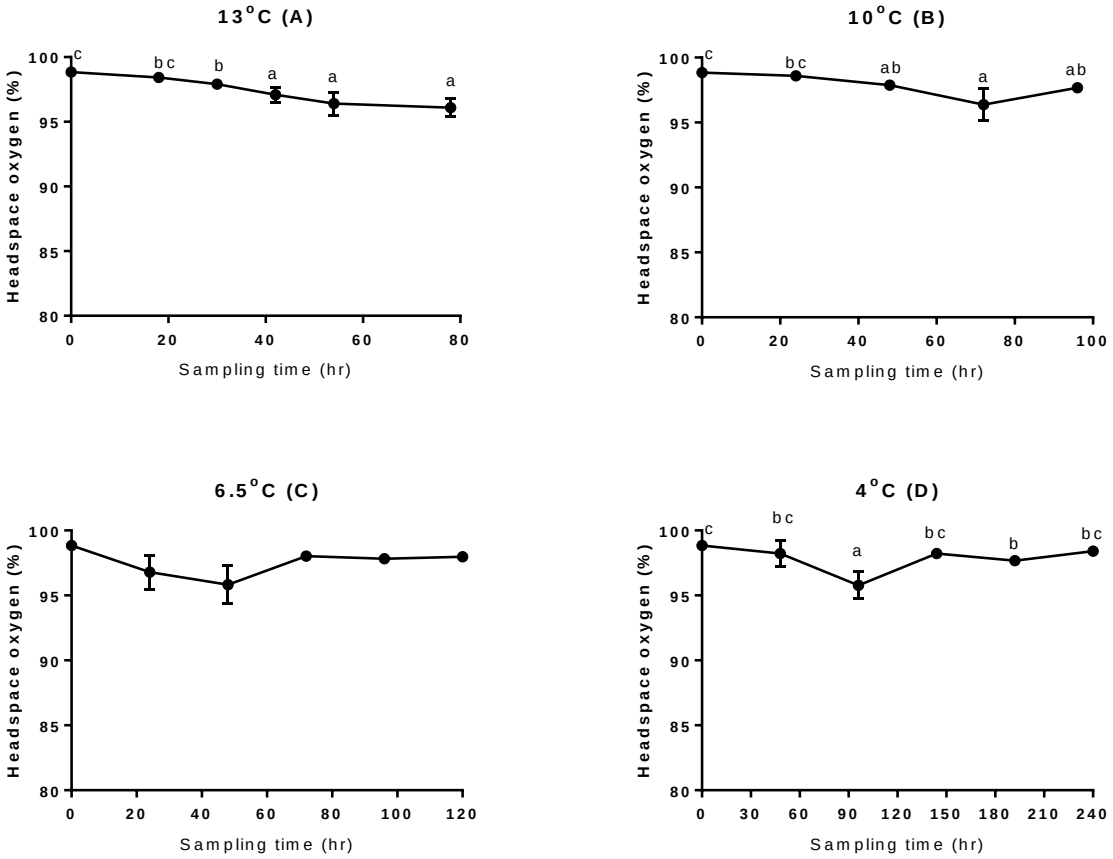


**Figure 2.3:** Headspace oxygen of samples stored at 13°C (A) for 78 hrs (3 days 6 hrs), 10˚C (B) for 96 hrs (4 days), 6.5˚C (C) for 120 hrs (5 days) and at 4˚C (D) for 240 hr (10 days). The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of headspace oxygen.

### 2.3.2 Physico-chemical analysis

#### 2.3.2.1 Dissolved oxygen in pouch water

There were significant ($p<0.05$) reductions in DO in the third samples of all the treatments (Figure 2.4).

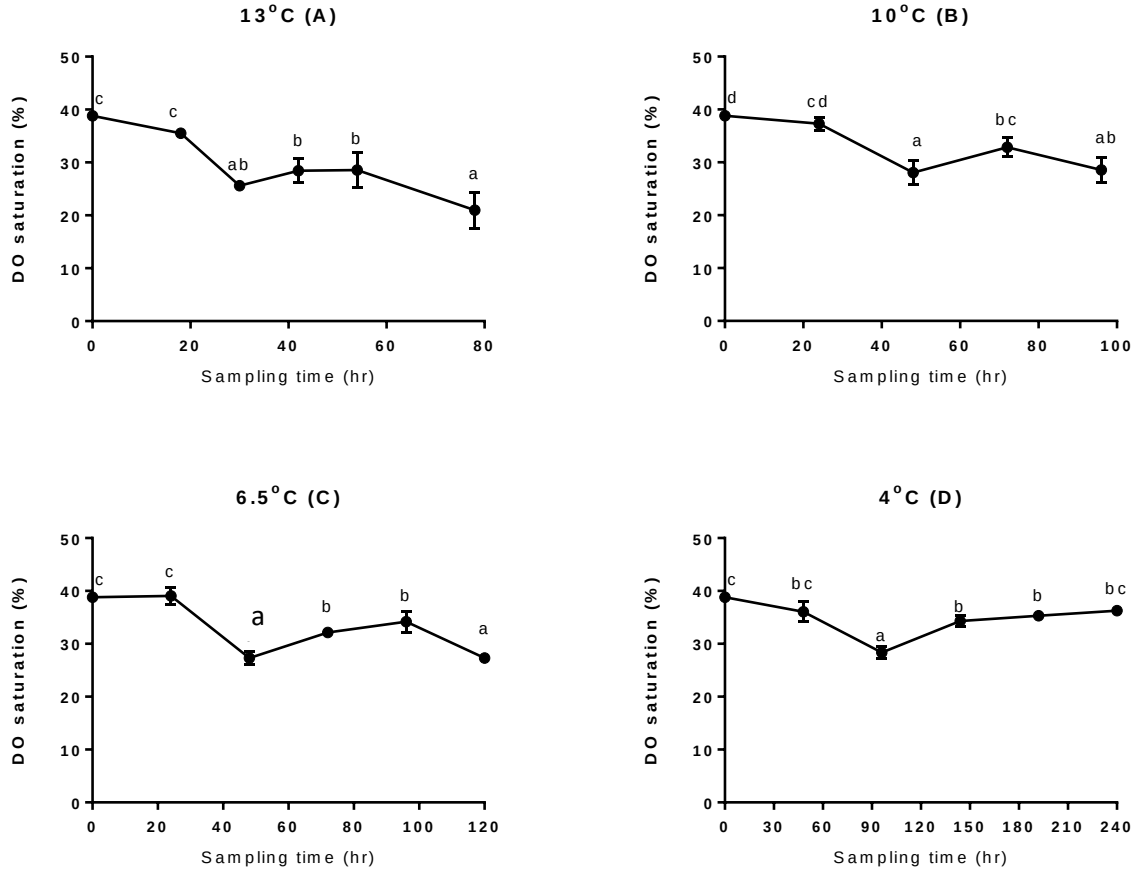


**Figure 2.4:** Dissolved oxygen (% saturation) of pouch water of samples stored at 13°C (A) for 78 hrs (3 days 6 hrs), 10˚C (B) for 96 hrs (4 days), 6.5˚C (C) for 120 hrs (5 days) and at 4˚C (D) for 240 hr (10 days). The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of DO.

A transient increase in DO was then observed in the 6.5°C and 10°C treatments. In the 4°C treatment, the DO was however, maintained until completion of the study.

#### 2.3.2.2 pH

The pH of pouch water added to each sample was investigated. In the samples stored at warmer temperatures (10°C and 13°C), it was observed that there was no significant difference ($p > 0.05$) in pH of pouch water (Figure 2.5).

At 30 hr of storage, the pH of samples stored at 13°C reduced to 6.68 ± 0.4 from 7.37 ± 0.17 at 18 hr storage period, increased to 7.18 ± 0.1 at 42 hr and remained constant during the remaining storage period. In the samples stored at 10°C, the pH reduced after 48 hr storage to 6.74 ± 0.05 from 7.04 ± 0.16. Increase in pH was observed at 72 hr storage. However, this was not significantly different ($p>0.05$) from 96 hr storage period. A similar pattern to 10°C was observed in the samples stored at 6.5°C.

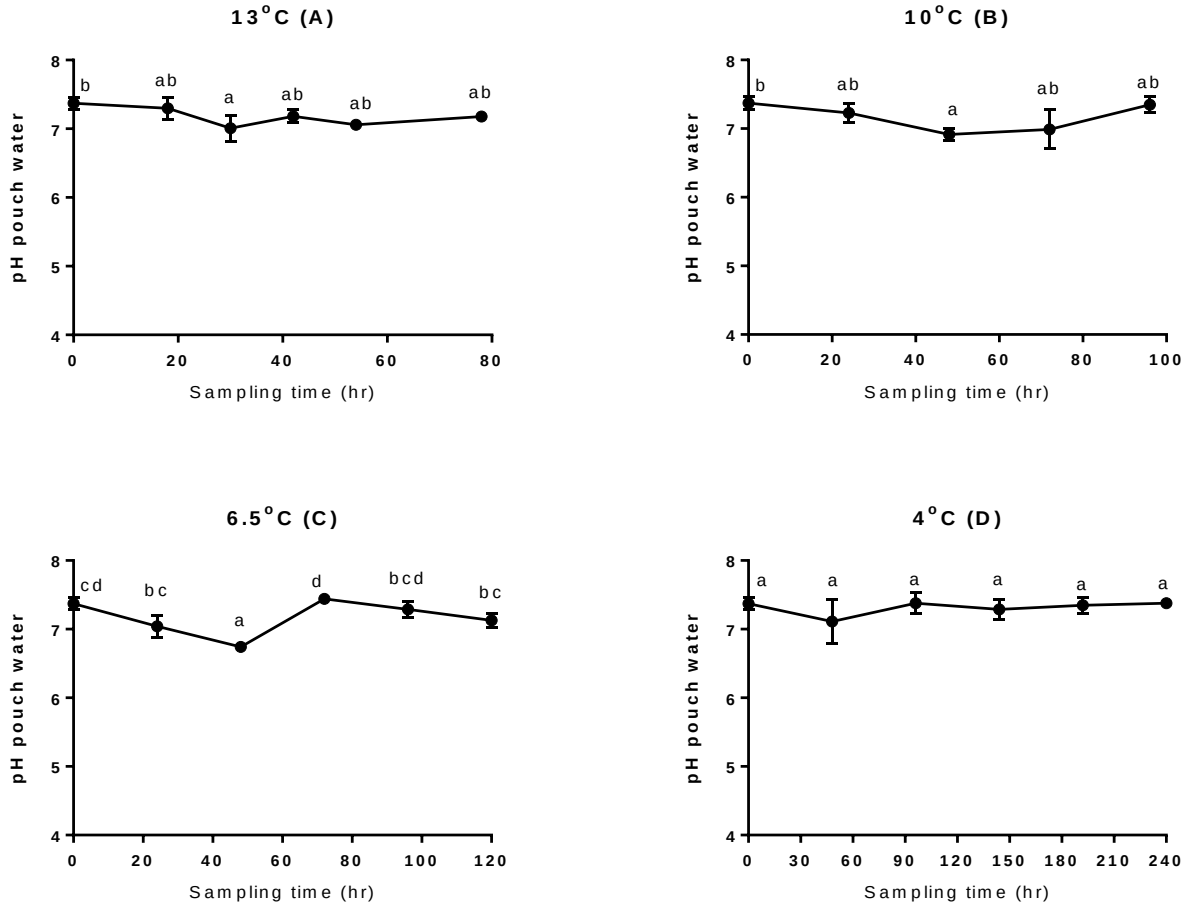


**Figure 2.5:** pH of pouch water of samples stored at 13°C (A) for 78 hrs (3 days 6 hrs), 10˚C (B) for 96 hrs (4 days), 6.5˚C (C) for 120 hrs (5 days) and at 4˚C (D) for 240 hr (10 days). The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of pH.

The pH of mussel meat of samples from each treatment was investigated and results presented in Figure 2.6.

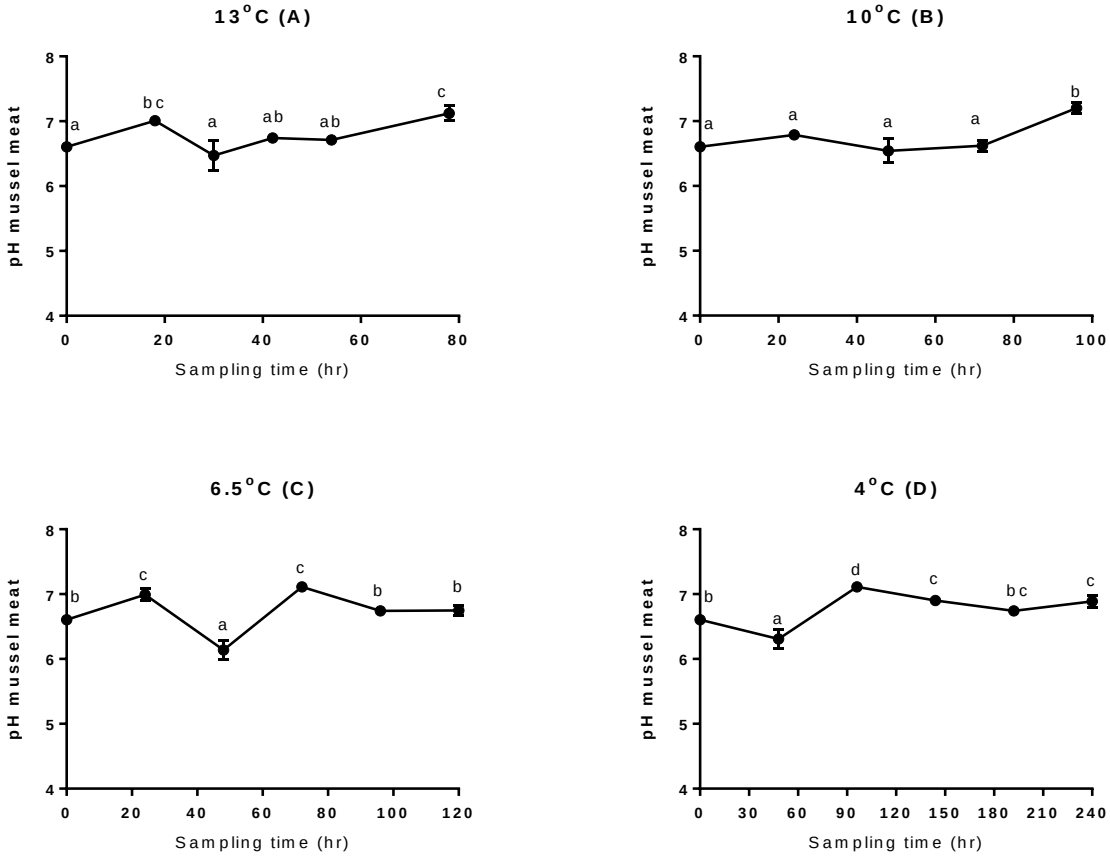


**Figure 2.6:** pH of mussel meat of samples stored at 13°C (A) for 78 hrs (3 days 6 hrs), 10˚C (B) for 96 hrs (4 days), 6.5˚C (C) for 120 hrs (5 days) and at 4˚C (D) for 240 hr (10 days). The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of pH.

### 2.3.3 Microbiological analysis

The mean TVC of pouch water of was below acceptable microbial limit of 6 log CFU $mL^{-1}$ (National Advisory Committee on Microbiological Criteria for Foods, 1992) at the start of the experiment (0 hr) (Figure 2.7). In the pouch water of samples stored at 4°C, it was observed that the lag phase was 75 hr compared to samples stored at other temperatures. For example, the TVC in samples stored at 6.5°C had 33 hr lag phase, while at 13°C it was 6.5 hr. No lag phase was observed in samples stored at 10 (Table 2.1). At 13°C, the TVC of pouch water at 78hr was significantly different ($p < 0.05$) from 0 and 18 hr.

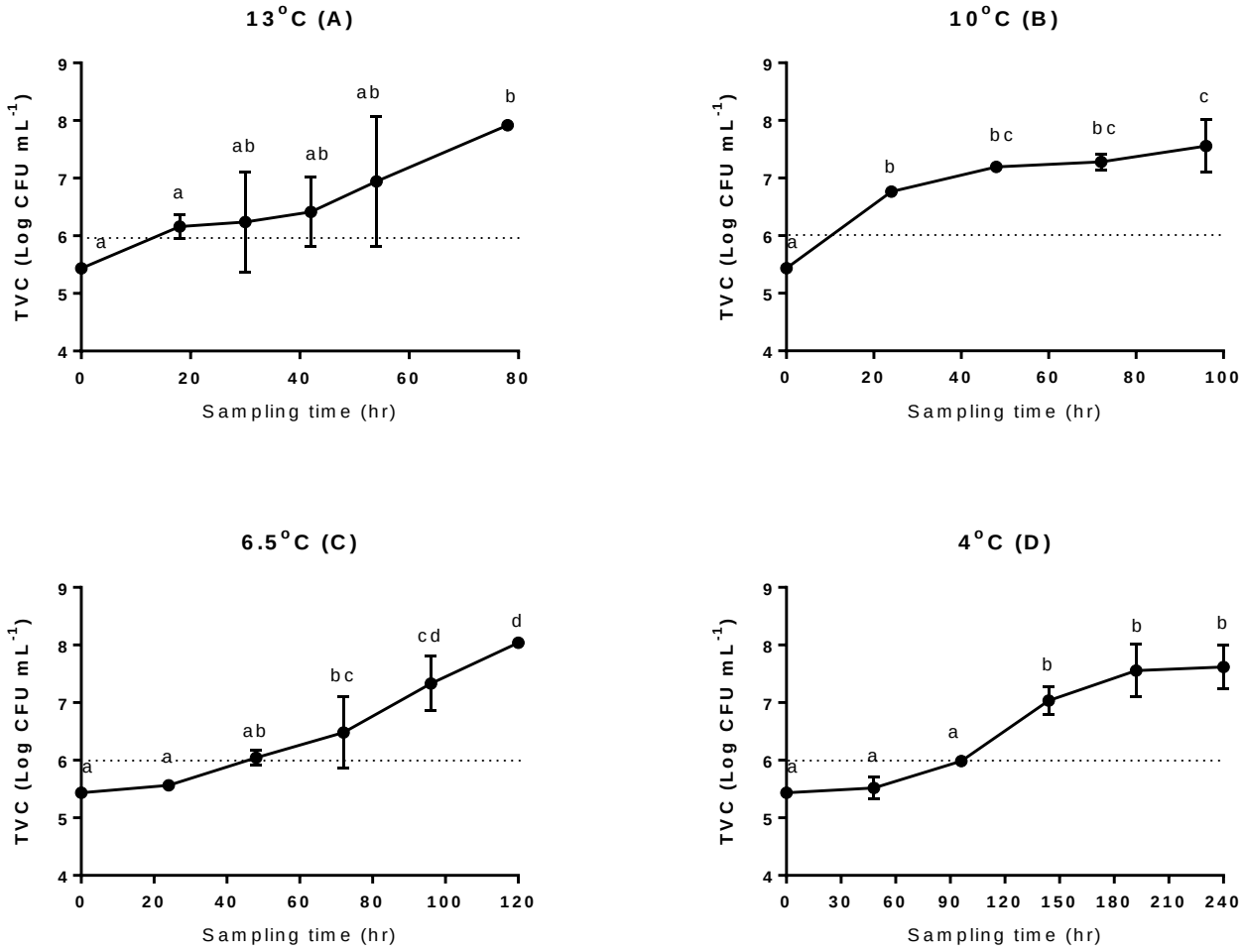


**Figure 2.7:** Total viable count – TVC (log CFU $mL^{-1}$) of pouch water of samples stored at 13°C (A) for 78 hrs (3 days 6 hrs), 10˚C (B) for 96 hrs (4 days), 6.5˚C (C) for 120 hrs (5 days) and at 4˚C (D) for 240 hr (10 days). The results represent mean and standard deviation of triplicate samples. The broken line represents acceptable microbial limit. The different superscripts denote significant differences ($p < 0.05$) in the means of TVC of pouch water.

**Table 2.1:** Fitting of the TVC of pouch water using Combase computer program.

| Storage temperature (°C) | Initial (CFU/mL) | Final (CFU/mL) | Maximum rate | Lag phase | $R^2$ | Standard error of fit | Model | Remark |
|---|---|---|---|---|---|---|---|---|
| 4 | 5.458 ± 0.115 | 7.598 ± 0.111 | 0.023 ± 0.005 | 75.307 ± 13.949 | 0.929 | 0.258 | Baranyi and Robert | Complete |
| 6.5 | 5.468 ± 0.146 | 8.037 ± 0.058 | 0.029 ± 0.004 | 33.191 ± 10.002 | 0.911 | 0.300 | Baranyi and Robert | No asymptote |
| 10 | 5.438 ± 0.141 | 7.345 ± 0.082 | 0.055 ± 0.009 | - | 0.907 | 0.243 | Baranyi and Robert | No lag |
| 13 | 5.496 ± 0.339 | 7.921 ± 0.017 | 0.032 ± 0.009 | 6.569 ± 18.163 | 0.611 | 0.595 | Baranyi and Robert | No asymptote |

**Table 2.2:** Fitting of the TVC of mussel meat using Combase computer program.

| Storage temperature (°C) | Initial (CFU/g) | Final (CFU/g) | Maximum rate | Lag phase | $R^2$ | Standard error of fit | Model | Remark |
|---|---|---|---|---|---|---|---|---|
| 4 | 5.336 ± 0.031 | 5.966 ± 0.212 | 0.003 ± 0.001 | 31.089 ± 22.009 | 0.979 | 0.031 | Baranyi and Robert | Complete |
| 6.5 | 5.34 ± 0.072 | 5.795 ± 0.036 | 0.016 ± 0.005 | - | 0.857 | 0.072 | Baranyi and Robert | No lag |
| 10 | 5.366 ± 0.051 | 5.853 ± 0.042 | 0.008 ± 0.002 | - | 0.934 | 0.055 | Baranyi and Robert | No lag |
| 13 | 5.397 ± 0.036 | 5.925 ± 0.021 | 0.017 ± 0.002 | - | 0.905 | 0.0639 | Baranyi and Robert | No lag |

The mean TVC of mussel meat of all treatments were below or at acceptable microbial limit of 6 log CFU $g^{-1}$ (Figure 2.8) throughout the storage experiment.

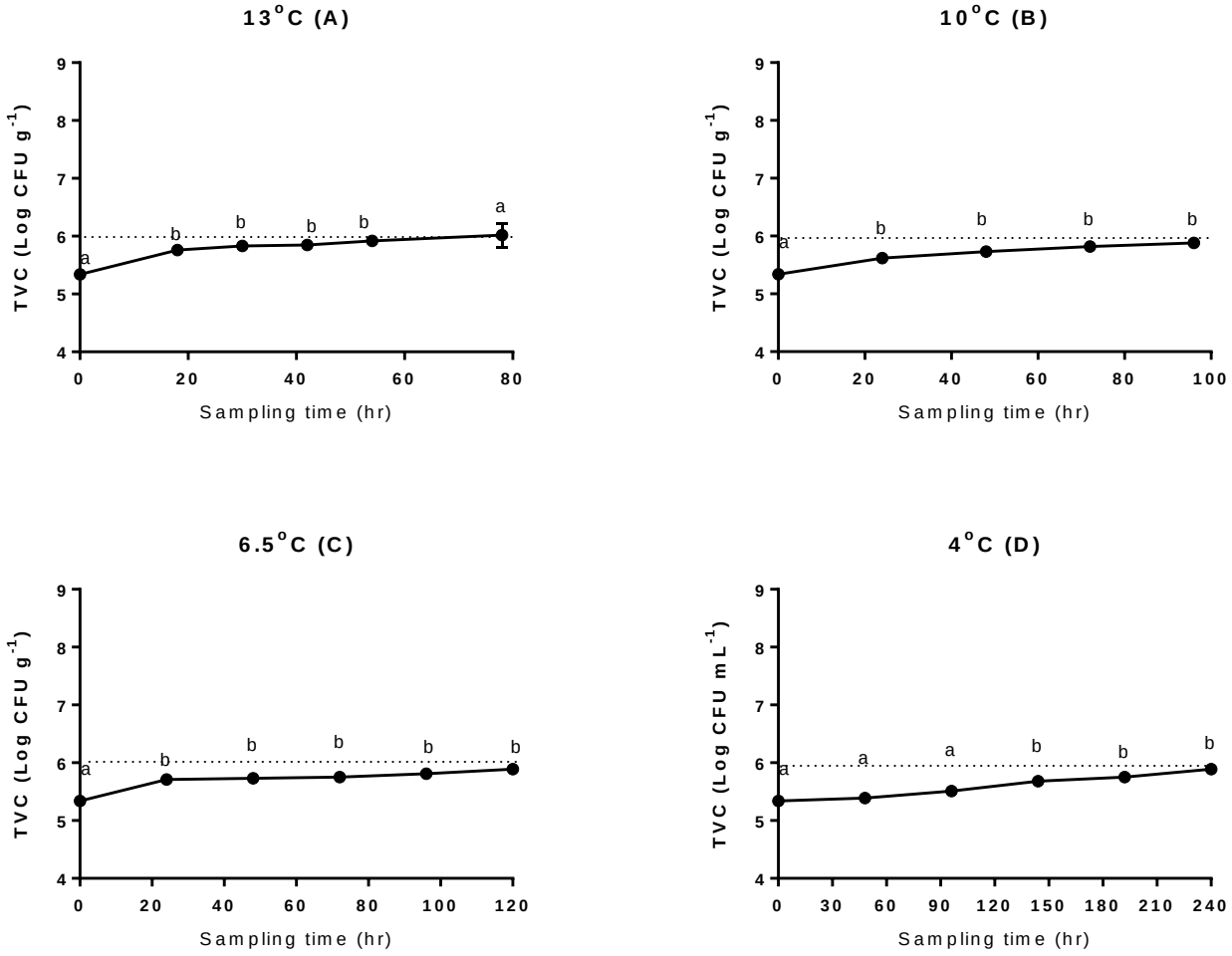


**Figure 2.8:** Total viable count – TVC (log CFU $g^{-1}$) of mussel meat of samples stored at 13°C (A) for 78 hrs (3 days 6 hrs), 10˚C (B) for 96 hrs (4 days), 6.5˚C (C) for 120 hrs (5 days) and at 4˚C (D) for 240 hr (10 days). The results represent mean and standard deviation of triplicate samples. The broken line represents acceptable microbial limit. The different superscripts denote significant differences ($p < 0.05$) in the means of TVC.

The mean TVC of all the samples stored at 4˚C, 6.5°C, 10°C and 13°C were below acceptable microbial limits at 0 hr. In comparison, the mean TVC of mussel meat was 5.34 – 6.02 log CFU $g^{-1}$ while the mean TVC of pouch water ranged between 5.44 – 8.04 log CFU $mL^{-1}$ within the

treatments across the storage period. In the mussel meat, only the samples stored at 4°C demonstrated a lag phase and showed a complete growth curve. However, the observed lag phase was 31 hr, less than half of the lag phase in pouch water at 4°C (Table 2.2). Microbial growth rates in the pouch water was greater than the microbial growth rate of mussel meat.

## 2.4 Phase 2: Optimisation of headspace oxygen

### 2.4.1 Headspace oxygen and $CO_2$

The results obtained from the study in Phase 1 showed that MAP live mussels packed with high (100%) initial headspace oxygen and stored at 4°C could retain freshness and quality of live mussels for at least 10 days post packaging.

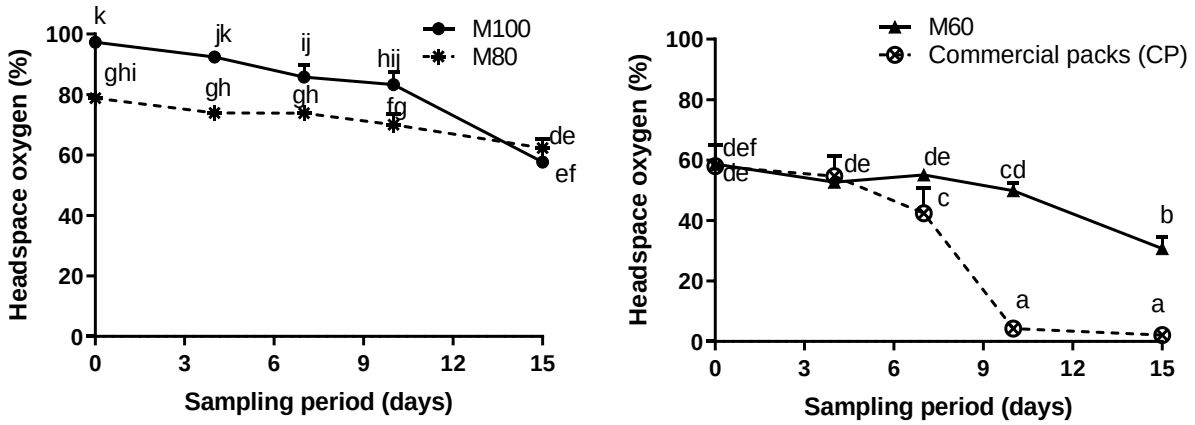


**Figure 2.9:** Headspace $O_2$ of samples packed with 100% (M100), 80% $O_2$ + 20 $N_2$ (M80), 60% $O_2$ + 40 $N_2$ (M60) initial headspace oxygen and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity.

However, there is need to optimise the initial headspace oxygen to ascertain the optimal range of high initial headspace. Samples of MAP live mussels after just 4 hr depuration were packed with fresh water in 100% (M100), 80% $O_2$ + 20% $N_2$ (M80), 60% $O_2$ + 40% $N_2$ (M60)

initial headspace oxygen (Figure 2.9). Commercial samples packed with 60% initial headspace oxygen were used as a reference. The results showed a decline in headspace $O_2$ with time and dramatic drop in the commercial samples at day 10. The headspace $CO_2$ was monitored alongside the headspace oxygen during the sampling days.

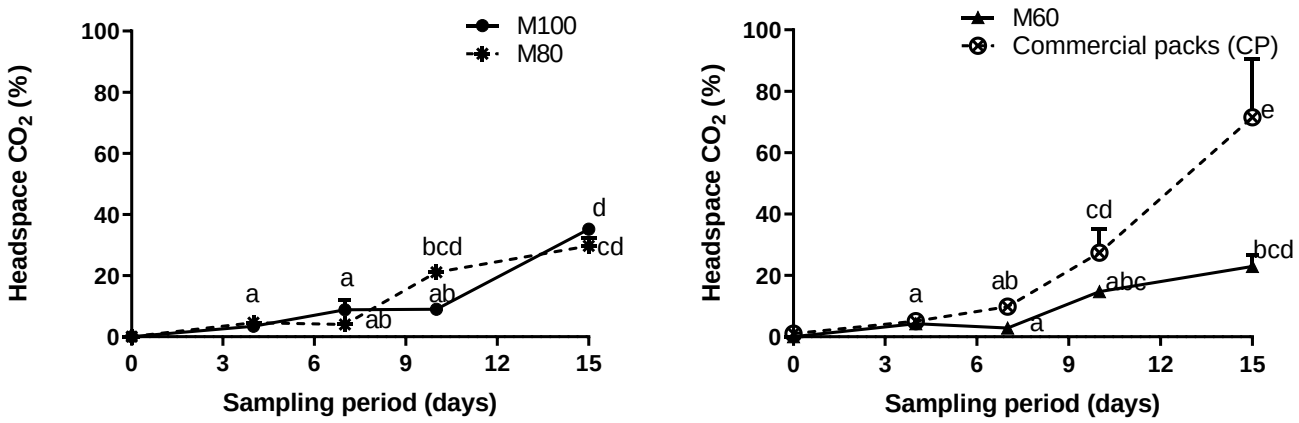


**Figure 2.10:** Headspace $CO_2$ (%) of samples packed with 100% (M100), 80% (M80), 60% (M60) initial headspace oxygen and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples stored at 4˚C stored for 15 days. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity.

No $CO_2$ was observed in any treatment aside from the commercial on day 0 (1.0 ± 0.5%) (Figure 2.10). There was no significant difference ($p>0.05$) in the $CO_2$ (2.21 – 11.2%) in all the treatments on day 4 and 7. No significant difference ($p>0.05$) was observed between M100, M80 and M60 on day 10 and 15. However, significant increase ($p<0.05$) in $CO_2$ of commercial packs was observed on day 10 (27.4 ± 7.6%) and 15 (71.5 ± 19.0%).

### 2.4.2 Physico-chemical analysis

#### 2.4.2.1 pH

The pH of pouch water added to the packs and pH of homogenized mussel meat were monitored (Figure 2.11). The pH varied in the range from 5.52 to 6.41 between all treatments and storage days. Little statistical variation was observed. A small increase in all the pH of all the treatments was observed on day 4.

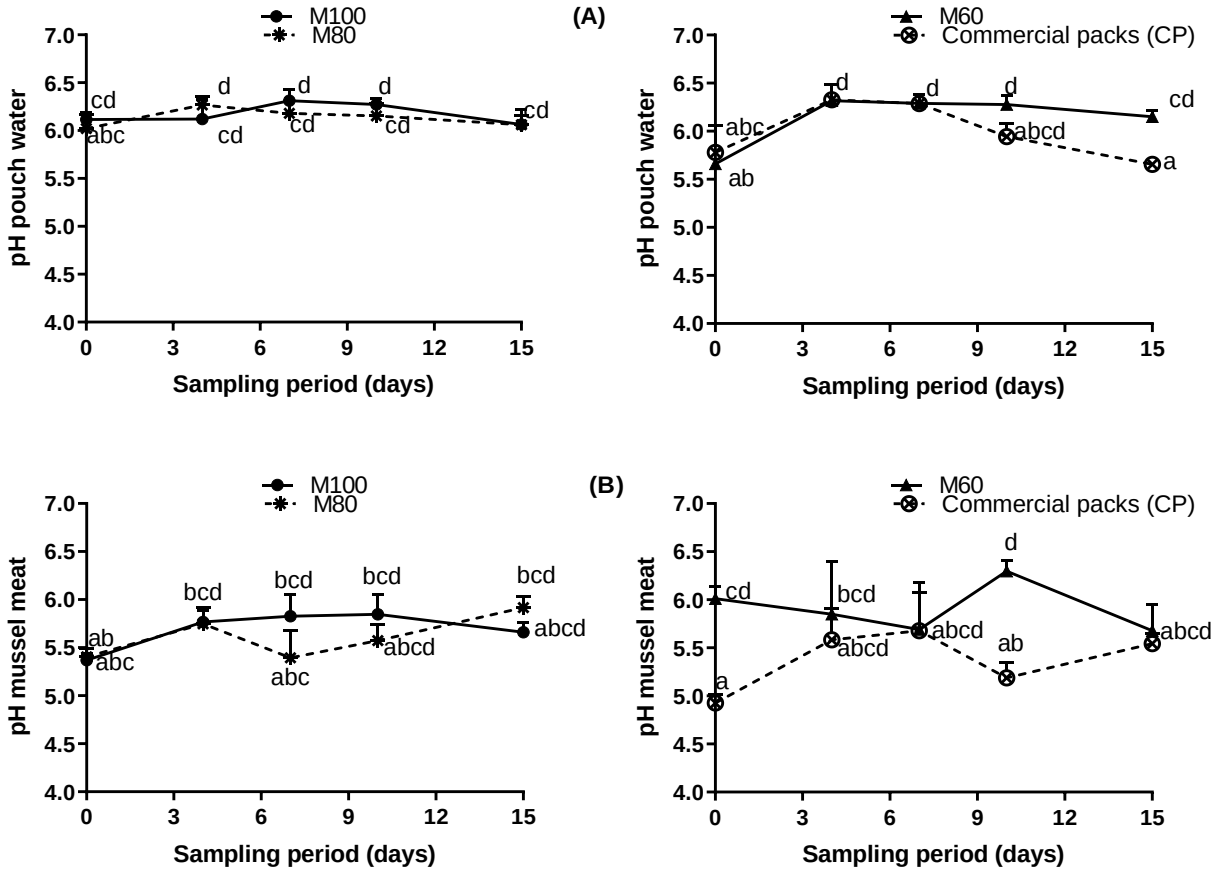


**Figure 2.11:** pH of pouch water (A) and mussel meat (B) of samples packed with 100% (M100), 80% (M80), 60% (M60) initial headspace oxygen and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity.

A significant decrease in the pH of commercial packs was observed on day 15 which was not observed in other treatments. The pH of homogenised mussel meat was also monitored (Figure 2.11). The pH of mussel meat was lower than that of pouch water varying in the range of 4.83 – 6.41, with little statistically significant difference ($p<0.05$).

### 2.4.2.2 Total ammonia nitrogen – TAN (mg $mL^{-1}$)

The mean TAN was below the detection limit (BLD = 0.5 mg $mL^{-1}$) in the treatments on day 0 aside from the commercial packs which was 3.5 mg $mL^{-1}$ (Figure 2.12). TAN was observed in all samples from day 4.

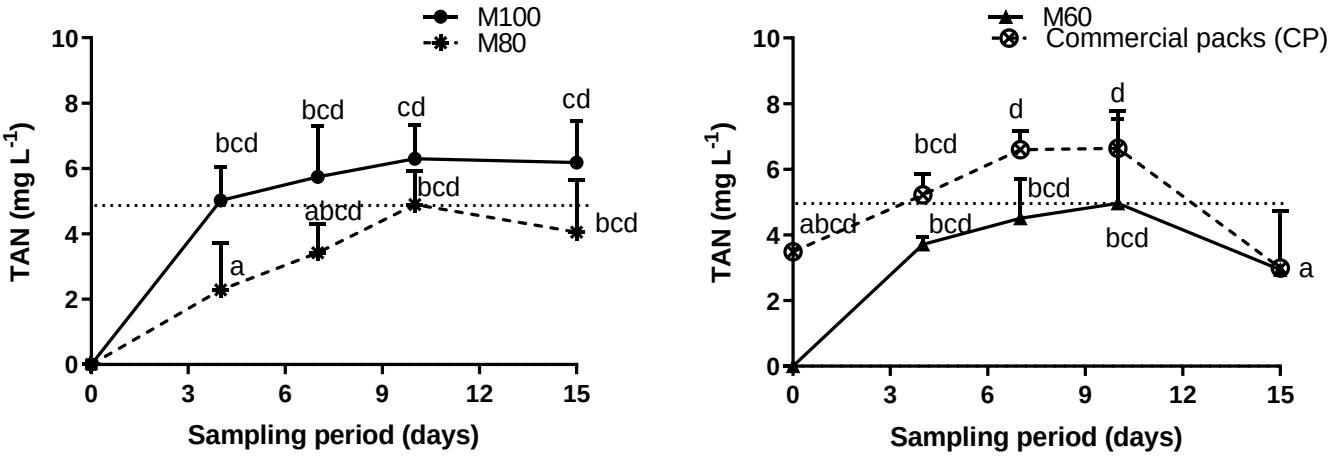


**Figure 2.12:** Total ammonia nitrogen (TAN) present in pouch water of samples packed with 100% (M100), 80% (M80), 60% (M60) initial headspace oxygen and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity. The broken line represents tolerance limit.

However, the mean TAN was below the tolerance limit (5 mg $mL^{-1}$) throughout the storage period in both M80 and M60. After initially increasing, TAN in the M60 and commercial samples treatment decreased on day 15. TAN in M100 exceeded the tolerance limit on day 7

but did not vary significantly thereafter. The mean TAN of the commercial packs was only below the tolerance limit on day 0 and 15.

### 2.4.2.3 Microbiological analysis

It was observed that the TVC increased significantly ($p < 0.05$) in all the treatments above microbial limit by day 7 and remained high to day 15 inclusive (Figure 2.13).

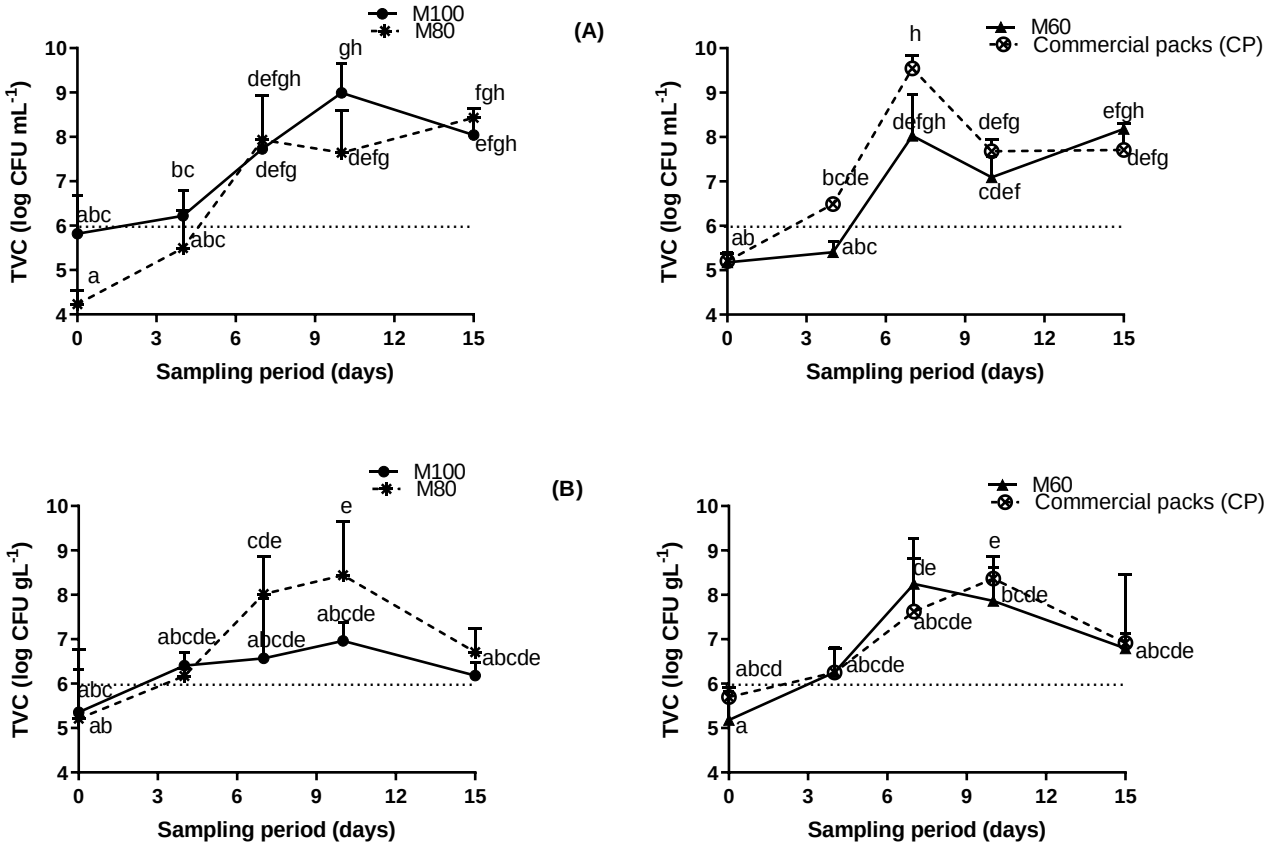


**Figure 2.13:** Total viable count – TVC of pouch water (log CFU $mL^{-1}$) (A) and mussel meat (log CFU $g^{-1}$) (B) of samples packed with 100% (M100), 80% (M80), 60% (M60) initial headspace oxygen and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity. The broken line represents acceptable microbial limit.

Objectionable smell was observed in both commercial packs and M60 samples on day 7 but wasn't observed until day 10 in both M100 and M80.

In comparison with pouch water, there was no significant difference ($p > 0.05$) in the mean TVC of mussel meat in the all treatments on day 0 and 4 and were below the recommended microbial limit (Figure 2.13). The mean TVC however, increased in all the treatments above microbial limit and by day 7 was significantly higher than day 0 counts for all 3 treatments and the commercial packs. No statistically significant difference ($p > 0.05$) was observed from day 7 to 15 between all three treatments suggesting the bacteria were in stationary phase. There was objectionable smell in the pouch water samples and the mussel meat on day 7 in M60 and commercial packs. This objectionable smell was observed on day 10 in M100 and M80 samples.

#### 2.4.2.4 Meat condition index

Meat condition index (MCI) was above marketable meat yield in all the treatments on day 0. The MCI was just at or above marketable meat yield on 4 and 7 in all the treatments. However, MCI of the commercial packs was below marketability on day 10 as were on day 15 (Figure 2.14).

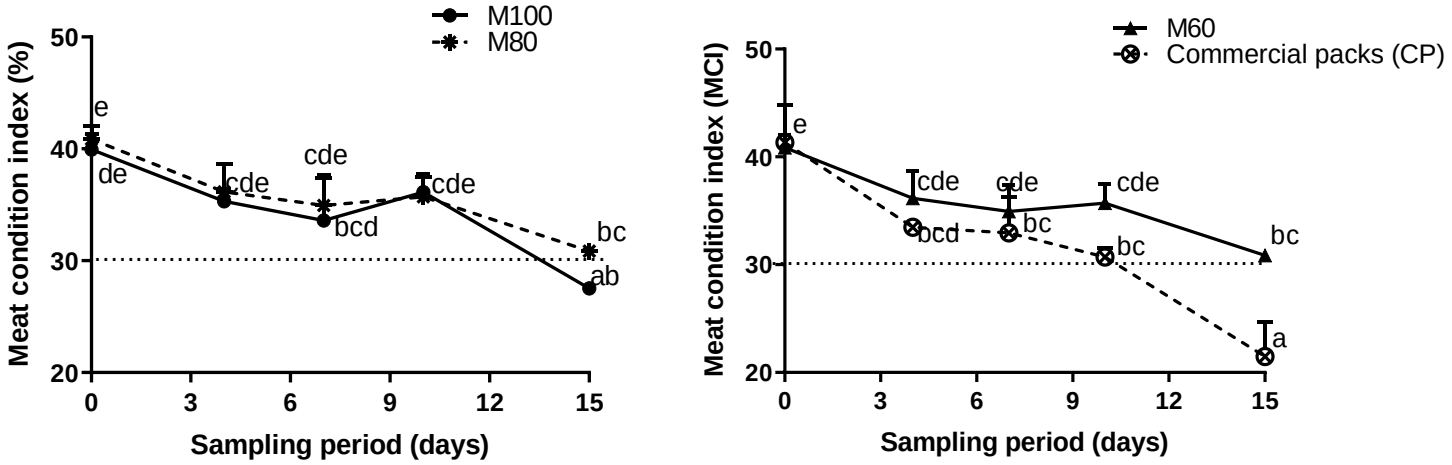


**Figure 2.14:** Meat condition index of cooked mussel meat of samples packed with 100% (M100), 80% (M80), 60% (M60) initial headspace oxygen and commercial packs stored at 4˚C for 15 days. Results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity. The broken line represents marketable meat yield.

There was no significant difference ($p > 0.05$) in MCI between the treatments on day 0 to 10. Physical observation of cooked mussel meat showed that samples M100 and M80 were palatable on day 10 while M60 and commercial packs were palatable on day 7 (Figure 2.15). Beyond day 7, the texture of cooked mussel meat in M60 and commercial packs became very soft and easily disintegrated.

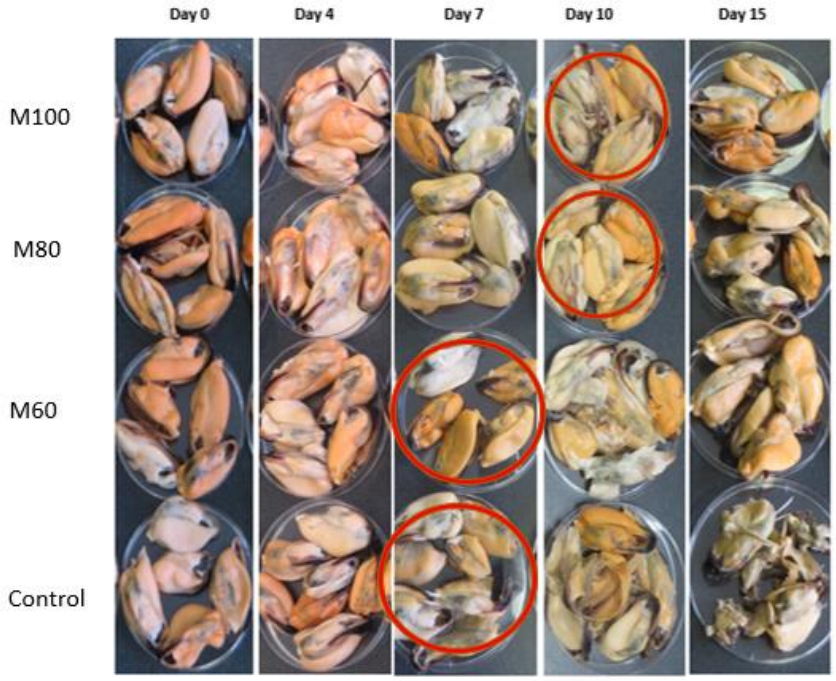


**Figure 2.15:** Cooked mussel meat of samples packed with 100% (M100), 80% (M80), 60% (M60) initial headspace oxygen and commercial packs stored at 4˚C for 15 days. The red circles indicate end of palatability of the cooked mussel meat.

## 2.5 Phase 3: Optimisation of depuration practices

The mean faecal collection during depuration is shown in Figure 2.17. Qualitatively, the amount of faecal matters increased with duration of depuration time.


**Figure 2.16:** Typical mussel faecal matters collected on a filter during depuration. The commercial packs did not have any mussels for 12 hr.

### 2.5.1 Headspace oxygen and $CO_2$

Significant differences ($p < 0.05$) started becoming obvious in the samples on day 4 (Figure 2.18) when the commercial packs reduced by about ≈ 17% to 69.76 ± 2.43%. There was a significant ($p < 0.05$) decrease in the headspace oxygen (%) in commercial samples between day 0, day 10 and 15. By day 15, there was a significant ($p < 0.05$) decrease in the headspace oxygen of commercial packs compared with samples depurated for 8 and 12 hr.

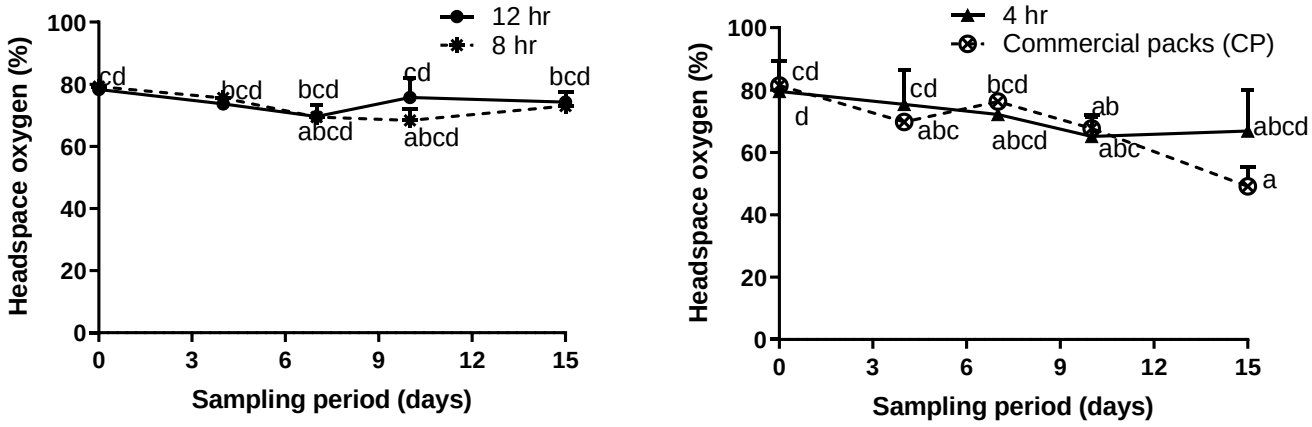


**Figure 2.17**: Headspace $O_2$ of samples depurated at 4 hr, 8 hr, 12 hr and commercial packs. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity.

As reported in phase 2 of this study, a gradual increase in headspace $CO_2$ during storage was observed in all the treatments (Figure 2.19). Headspace $CO_2$ significantly increased over time from day 0 to day 15 (0 – 25.9%). The highest was observed in the commercial packs.

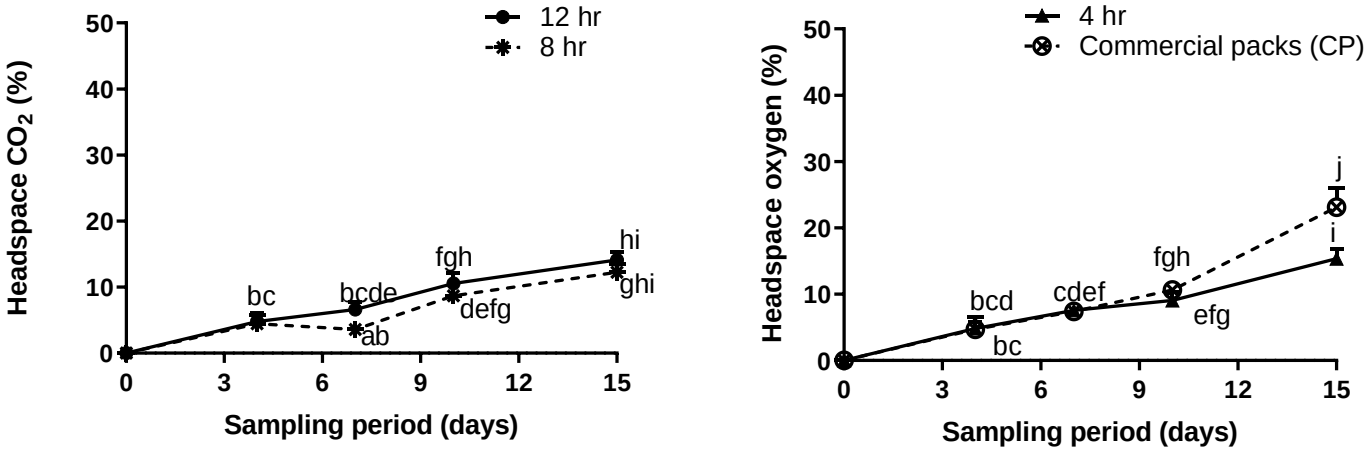


**Figure 2.18**: Headspace $CO_2$ of samples depurated at 4 hr, 8 hr, 12 hr and commercial packs. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity.

### 2.5.2 Physico-chemical analysis

#### 2.5.2.1 pH

There were significant differences ($p < 0.05$) in pH (5.63 – 6.79) of pouch water within the treatments across the storage days (Figure 2.20).

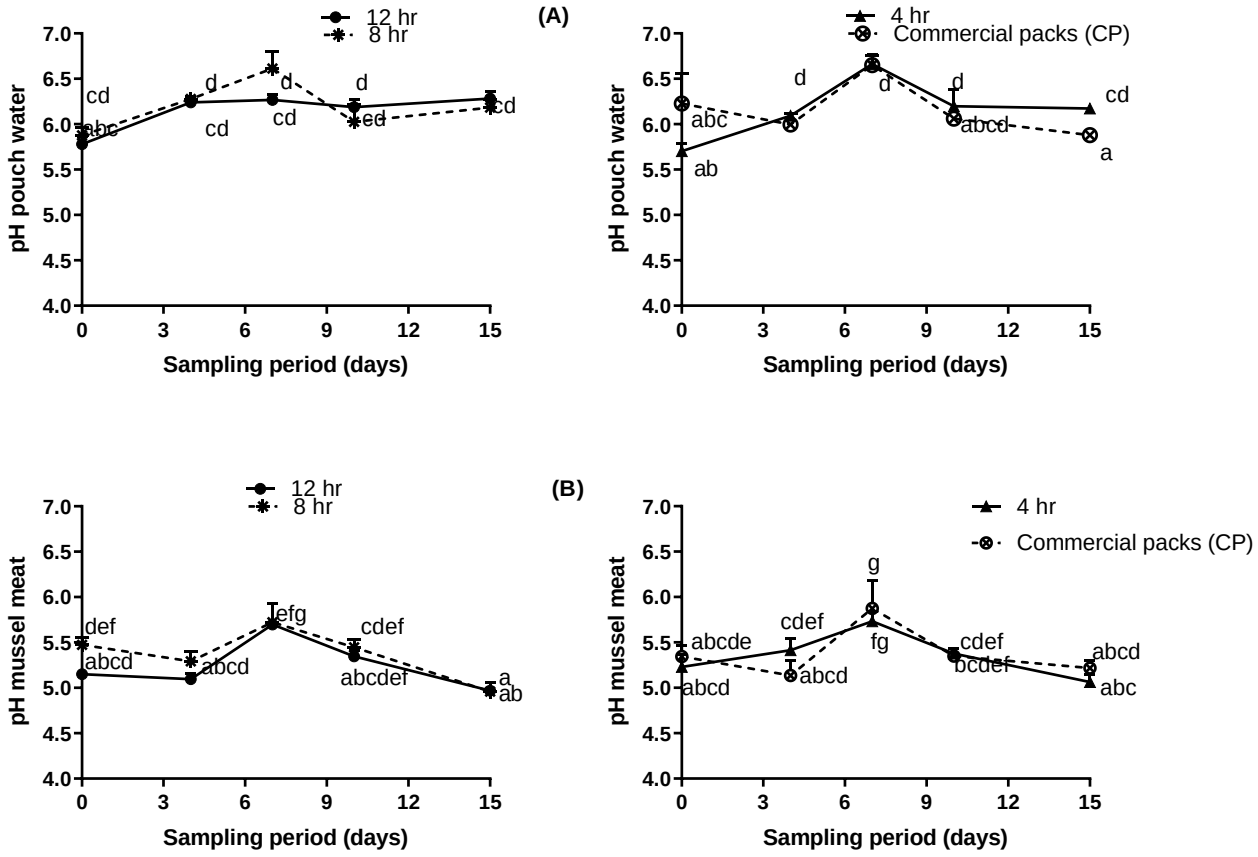


**Figure 2.19**: pH of pouch water (A) and mussel meat (B) of samples depurated at 4 hr, 8 hr, 12 hr and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity.

The commercial packs did show small but significant difference ($p < 0.05$) compared to 4 and 12 hr treatments on day 0; and 12 hr on day 15. The pH of mussel meat (4.9 – 6.19) was lower than that of pouch water in all the treatments (Figure 2.20). When compared with pH of pouch

water (5.67 – 6.27), the mean pH of mussel meat ranged between 5.13 – 5.50 on day 0. On day 15, the mean pH of mussel meat was 4.94 – 5.10 compared to the mean pH of pouch water (5.99 – 6.20).

### 2.5.2.2 Total ammonia nitrogen – TAN (mg $mL^{-1}$)

No TAN was observed in any treatments on day 0, but the commercial packs did have some (4.07 mg $mL^{-1}$) (Figure 2.21). TAN was only detected in commercial packs samples from day 0 to day 7.

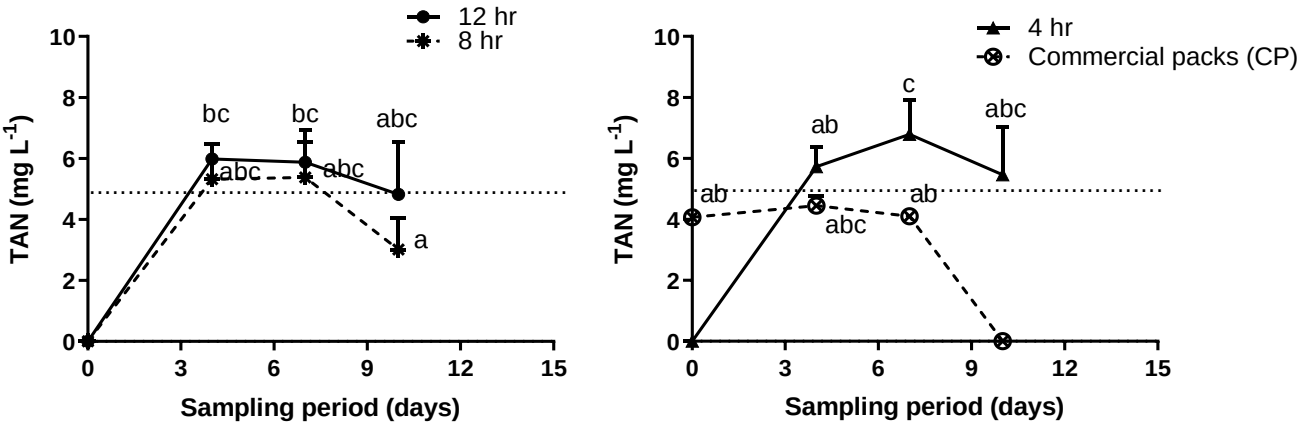


**Figure 2.20**: Total ammonia nitrogen (TAN) of pouch water of samples depurated at 4 hr, 8 hr, 12 hr and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. NS = no significant difference. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity. The broken line represents acceptable limit.

However, TAN was observed in all samples from day 4 to day 10 and was above tolerance limit (5 mg $mL^{-1}$) in 4, 8 and 12 hr treatments for day 4 and day 7. TAN was not detected in the commercial packs on day 10 of only and in all samples of all the treatments on day 15. This

was because the mussel meat had disintegrated and became a slurry in the packs, which made it difficult to detect the presence of TAN in the samples.

### 2.5.3 Microbiological analysis

The mean TVC of pouch water in all treatments was below acceptable microbial limits aside from the commercial packs on day 0 which was significantly higher than the other treatments (Figure 2.22). The mean TVC of commercial packs was both above the acceptable microbial limit (6 log CFU $mL^{-1}$) and significantly higher than the other three treatments on day 0, 4, 10 and 15. On day 10, the commercial packs differ significantly from the treatments. There was no significant difference ($p > 0.05$) in the mean TVC between all treatments on day 7. The depuration treatments were above the limit from day 7 and remained so for the remaining duration of the experiment.

In the mussel meat, all treatments and commercial packs were above microbial limit on day 7 but treatments significantly decreased ($p < 0.05$) subsequently on day 10 to about the limit, after which the 12 h treatment TVC rebounded. The overall mean TVC of pouch water (6.62 log CFU $mL^{-1}$) was not significantly different ($p > 0.05$) from the TVC of mussel meat (6.34 log CFU $g^{-1}$).

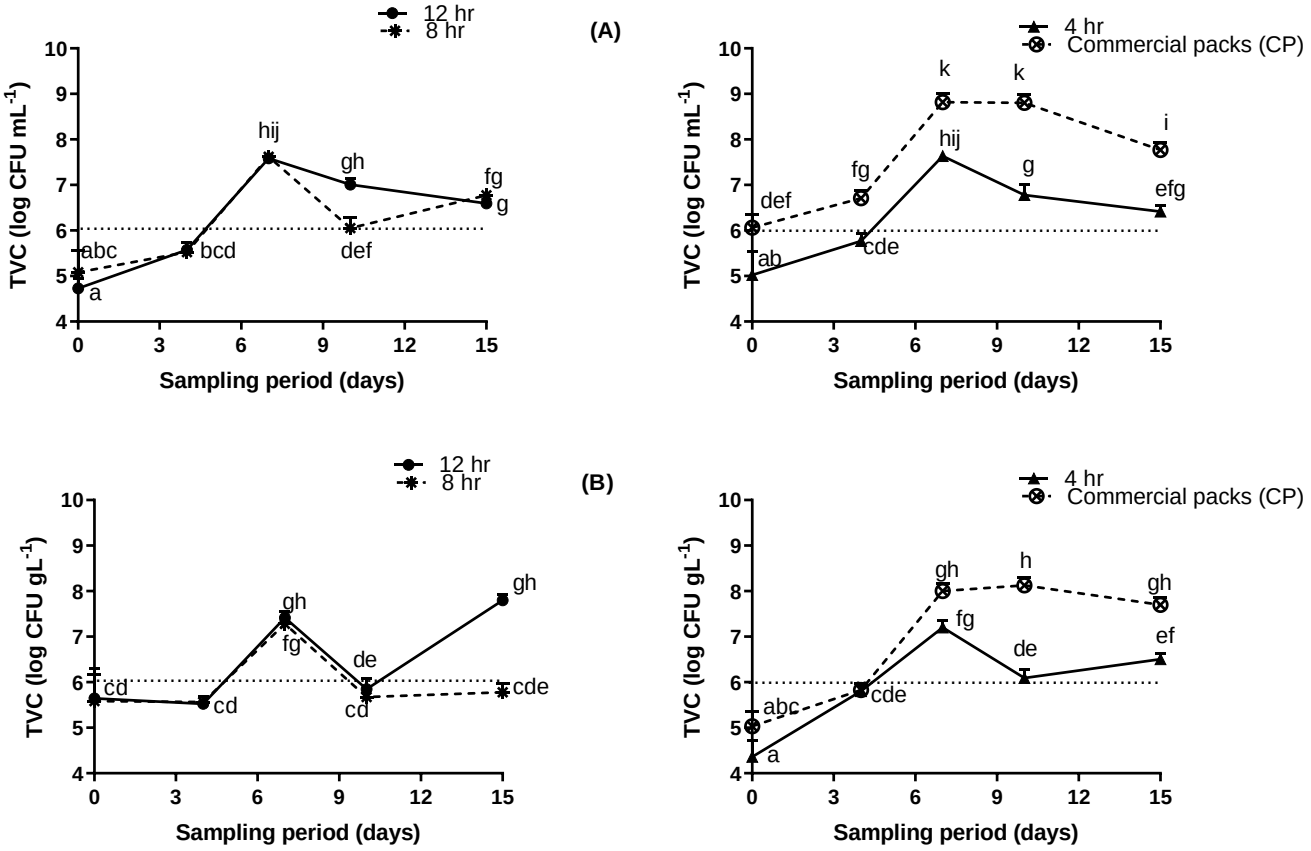


**Figure 2.21**: Total viable count – TVC of pouch water (log CFU $mL^{-1}$) (A) and mussel meat (log CFU $g^{-1}$) (B) of samples depurated at 4 hr, 8 hr, 12 hr and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity. The broken line represents acceptable microbial limit.

### 2.5.4 Meat condition index

It was observed that MCI in all treatment was within market range (MCI = 30%) although it reduced significantly ($p < 0.05$) in the commercial packs on day 15 (Figure 2.23).

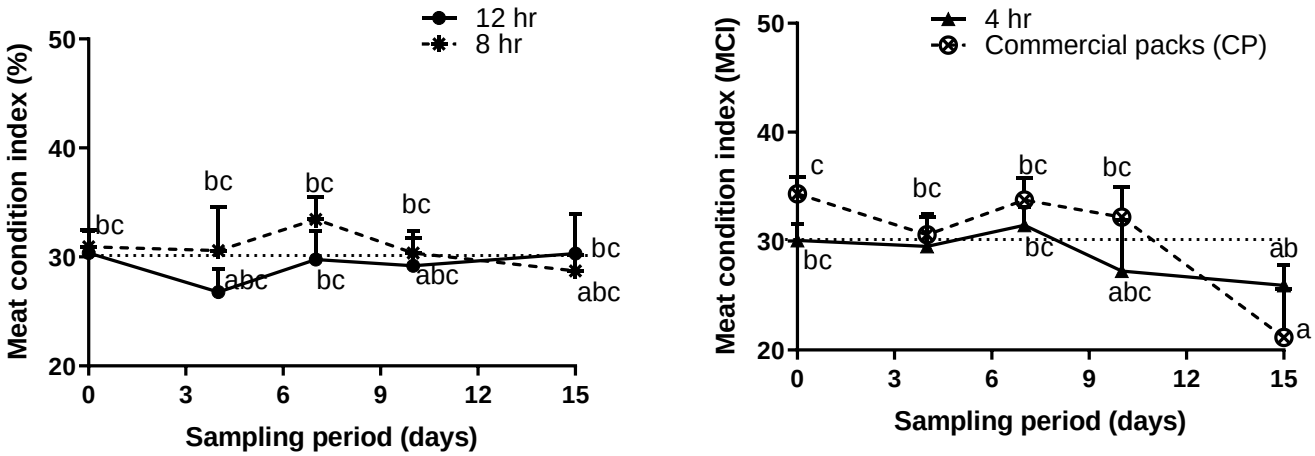


**Figure 2.22**: Meat condition index of samples depurated at 4 hr, 8 hr, 12 hr and commercial packs stored at 4˚C for 15 days. The results represent mean and standard deviation of triplicate samples. The different superscripts denote significant differences ($p < 0.05$) in the means of all values across both graphs. Data were only separated to improve visual clarity. The broken line represents marketable meat yield.

Raw mussel meat was visually observed for any degradation (Figure 2.24) and visual acceptability was assessed after cooking the mussels (Figure 2.25). It was observed that raw mussel meat became soft, disintegrated and unacceptable on day 10 in both 4 hr depuration and commercial packs. It also became disintegrated and unacceptable on day 15 for both 12 and 8 hr depuration. Similar results were observed in cooked mussel meat (Figure 2.25).


**Figure 2.23**: Raw mussel meat of samples depurated for 4 hr, 8 hr, 12 hr and commercial packs stored at 4°C for 15 days. The red circle indicates days of onset of unpalatability.


**Figure 2.24**: Cooked mussel meat of samples depurated for 4 hr, 8 hr, 12 hr and commercial packs stored at 4°C for 15 days. The red circle indicates days of onset of unpalatability.

## 2.6 Discussion

The shelf-life of air-packed live mussels without added pouch water stored at 2 – 3°C was reported to be 3 – 4 days while live mussel packed with high initial oxygen (75%) without added pouch water had shelf-life of 6 days (Pastoriza et al., 2004). However, current industrial practice indicates that the shelf-life of live mussels in modified atmosphere packaging (MAP) is limited to 7 – 10 days due to either or both autolytic degradation of mussels and bacterial growth in the pouch water. The result was an off-smell that could render the product unacceptable. To be kept alive during storage, mussels require an adequate supply of oxygen (Pastoriza et al., 2004).

Storage temperature during the supply chain of live mussels will affect quality and shelf-life through its influence on mussel respiration, bacterial growth rates and bacterial respiration (Bernardez and Pastoriza, 2011). The gastrointestinal tract (GIT) is a significant source of transient and indigenous bacteria as well as organic carbon through faeces. Transient GIT bacteria can be reduced by depuration. In some studies, depuration was carried out for 48 hr (Barrento et al., 2013) or more (Novaczek et al., 1992). None of these studies stated the effectiveness of depuration in removing initial microbial load. Similarly, long duration of depuration is not commercially viable due to cost. Thus, there is a need to establish the most suitable and cost-effective depuration process and duration that lowers the bacterial load as much as possible, without compromising either mussel condition or farm economics. This study investigated optimization of post-harvest storage conditions required for retaining

freshness and palatable quality of MAP live mussels. Storage temperature, initial headspace oxygen and duration of depuration were examined.

### 2.6.1 Optimisation of storage temperature of MAP live mussels.

Most studies on shelf-life of mussels are based on post mortem (shucked mussels) rather than live mussels (Caglak et al., 2008; Erkan, 2005). Keeping mussels alive during storage requires a good source of oxygen, thus the animals need to be packed live in gas-tight containers. Mussels are known to have an innate immune system that can fight against pathogenic and non-pathogenic bacteria and viruses. Despite this, proliferation of indigenous microbes in mussels during post-harvest handling can easily result in spoilage and a reduced shelf-life. Bernardez and Pastoriza (2013) reported that 7°C is enough to favour higher microbial growth in live mussels.

Since water was added to the pouches in this study to mimic commercial practices, part of the oxygen added for mussel survival would diffuse into the pouch water as dissolved oxygen. Low dissolved oxygen also known as hypoxia could stress aquatic animals resulting in mortality (Wang and Widdows, 1991). Dissolved oxygen (DO) measured in mg $L^{-1}$ is an indicator of water quality and aquatic animals' health in aquaculture (Caraco et al., 2000). (Barrento et al., 2014) reported that mussels consume oxygen at high rates (7.5- 12.2 µmol $g^{-1}$ $h^{-1}$) within 6 hrs of immersion in water and decreased afterwards to 4.0 ± 0.9 µmol $g^{-1}$ $h^{-1}$. Their results could not be compared to this present study because a flow through system was used in their study while a closed system was used in this present study. More so, fresh water

was added to the pouch in this study to keep the mussels hydrated. However, the mussels would not open and pump water as they would in the study of (Barrento et al., 2013) Response of mussels to DO depends on factors such as feeding, oxygen uptake, and heat dissipation (Wang and Widdows, 1991).

The pH of pouch water in all the samples was observed to be higher than that of the mussel meat. When bivalve shellfish die, fatty acid and protein are broken down causing increased pH (5.9 – 6.57) that can lead to increased microbial proliferation. Increased pH could also be because of deterioration and production of volatiles such as trimethylamine produced by spoilage bacteria (Goulas and Kontominas, 2005). Although there was not much significant difference in the pH of both pouch water and mussel meat in all the treatments at the early stage of storage, increases in pH were observed towards the end of the storage days.

Microbes play significant roles in spoilage of seafood because they can use seafood muscle as substrate for growth. Increased microbial metabolism is thereby likely to increase deterioration of the quality of the seafood. The period of lag phase increased (6.5 – 75 hr) with decreased storage temperature in the pouch water while lag phase was reported only at 4°C in the mussel meat. No lag phase in both the mussel meat and pouch water at 10°C storage. As observed in this study, research has shown that low storage temperature increases the lag phase of microbes thereby extending the shelf-life and low temperature decreases growth rates (Alfaro and Hernandez, 2013; Boziaris et al., 2013). It was however observed that the lag phase of mussel meat (31 hr) was approximately half that of pouch water (75 hr) in the samples stored at 4°C. This implies that the pouch water provided suitable

biological medium for microbial growth. The TVC was observed to be at or below microbial limit (6 log CFU /g) at 0 hr in all the treatments in mussel meat. No objectionable smell was observed in any of the treatments in this study in phase 1. This study showed that the storage of MAP live mussels at 4°C prolong the lag phase and decrease the growth rate. However, the product was not tasted. Similarly, temperature below 4°C was not investigated because such temperature will be too cold for the mussels thereby resulting into death or rapid loss of sensory quality of the mussels.

### 2.6.2 Optimisation of headspace oxygen of MAP live mussels.

As expected, the headspace oxygen concentrations in the M100 and M80 treatments were significantly higher than those of the M60 treatment and the commercial packs throughout the experiment. Consumption of oxygen due to metabolic activities of the mussels, diffusion of oxygen into the pouch water and uptake by microbes resulted in a decrease in headspace oxygen.

The headspace oxygen was significantly different from each other in M100 and M80 throughout the experiment apart from day 15. By day 15, both M100 and M80 had ≈ 60% oxygen, insignificantly different from the initial oxygen in M60, but significantly higher than the oxygen in M60 after 15 days. The drastic decline in headspace oxygen in the commercial packs was not observed in other treatments. This could be attributed to the fact that commercial samples were packed earlier (12 hr) before other treatments and because there were between 41 and 48 individual mussels in the commercial pack samples compared to 6

– 8 mussels in the treatments. The laboratory-packed mussels were in proportionally smaller containers.

Higher DO increases oxygen availability for mussel and bacterial respiration. This results in increased metabolic activity and excretion of ammonia by the mussels. Similarly, it causes reduction of available oxygen which if it continues overtime, there will be mortality of mussels. On day 10, which is the commercial shelf-life day, 92% decrease in oxygen was observed in the commercial packs. This was because of the effect of diffusion which goes faster than in the commercial packs (60% $O_2$) which is strictly limited by the concentration at the source (headspace) and the receiving part (the pouch water). It would be expected that diffusion would be faster from a higher concentration than from a lower concentration. Alternatively, as the commercial packs and M60 started out the same, then the commercial packs may be consuming oxygen faster, so that the rate of decline was higher than in the M60. The commercial packs (60% oxygen) were of large sizes (volume to product ratio - 1kg) compared to laboratory packs (M80 and M60). Although the commercial packs had 60% oxygen, the rate of diffusion from headspace to the pouch water was faster, in addition to the high oxygen consumption by the high number of mussels (> 40 mussels) and bacterial respiration. M80 has enough oxygen to continually supply demand, M60 had insufficient to meet demand and decline in oxygen while the CP (60%) had declined oxygen due to a high number of (> 40 mussels) of mussels, increased respiration loss and high diffusion rate.

Although there have been a few studies on quality of MAP live mussels, none monitored oxygen decline during storage. Monitoring the decline gives an indicator of what might be causing oxygen consumption (mussels vs microbes) by looking at the $O_2$ decline in comparison

to other parameters (like TVC and TAN). For example, Pastoriza et al. (2004) studied use of high initial headspace oxygen (75%) to extend shelf-life of live mussels stored at 2 – 3 °C to 6 days compared to air- packed live mussels with 3 - 4 days shelf-life at the same storage temperature. However, they did not monitor decline in oxygen over the storage time. Similarly, Bernardez and Pastoriza (2011) assessed the impact of high oxygen (75% and 85%) and mussel size on quality of live mussels stored at 1 - 3°C, yet the decline in the headspace oxygen was not monitored. Bernardez and Pastoriza (2013) investigated the effect of 83% and 20% initial headspace oxygen concentrations and storage temperatures (2 ± 1°C or 7 ± 1°C ) on quality of live mussels, but the decline in the headspace oxygen during storage was not monitored during the experimentation.

As expected, $CO_2$ (from mussels and bacterial respiration) increased with storage days in all the treatments in this current study. There was no significant difference in $CO_2$ concentration between the treatments on days 0, 4 and 7. On day 15, a significant increase in $CO_2$ to 35.2 ± 1.75% occurred in M100 packs and to 29.7 ± 2.81% in M80 packs. M60 increased by 22.9% while commercial packs samples had 71.5% $CO_2$. The increased $CO_2$ observed in M60 could cause a shift in spoilage microbiota as $CO_2$ is inhibitory to some bacteria and the high concentration observed in the commercial packs on day 15 might cause a change in the spoilage microbiota to $CO_2$ resistant bacteria such as *Photobacterium phosphoreum* (Timo et al., 2016). Similarly, due to the system being sealed and static, oxygen only moves via diffusion (which is slower than metabolic rates), hence, there is possibility of anoxic micro zones in the water around the mussels. Consumption of oxygen due to metabolic activities of the mussels,

diffusion of oxygen into the pouch water and microbial metabolism resulted in decreased headspace oxygen and increased $CO_2$ release.

Pastoriza et al. (2004) reported 12.1% headspace $CO_2$ on day 4 in live mussels packed with 75% initial oxygen and stored at 2 - 3°C for 6 days. In a study involving MAP live clams, it was reported that rate of respiration increases with increase in $CO_2$ (Ho et al., 1997). A similar result was observed in live clams packed with 70% initial oxygen and stored at > 5°C for 6 days (Goncalves et al., 2009). They observed 11% $CO_2$ on day 6. Studies on the rate of respiration in MAP live mussels have yet to be carried out, therefore, it was difficult to correlate exactly with the rate of oxygen consumption with the increment of $CO_2$ during cold temperature storage but qualitatively, it is likely that both mussels and microbes are respiring.

The metabolism of seafood glycogen increases lactic acid accumulation that could reduce the pH of seafood. According to (Aru et al., 2016b) the changes in pH of shucked mussels during cold storage could be used to assess quality. However, live mussels are expected to have pH buffering capacity except when morbid. The pH of fresh and good quality mussels ranged between 6 and 7 (Colby et al., 1993; Khan et al., 2005). However, this was contrary to the result obtained in this study (pH = 4.9 – 6.19). Aru et al. (2016b) stated that pH 5.77 indicates degradation of stored glycogen during cold storage of shucked mussels at 0 – 4°C. When their results were compared with this present study, it was observed that on day 0 in this study, mussels have started utilizing stored glycogen as an energy source in all the treatments because they were not fed throughout the experimental period, and they require energy for metabolic activities during the storage period. Hence, they could only obtain this energy from

the reserved energy (glycogen). There is need to study the change in glycogen content with storage to ascertain the claims of Aru et al. (2016b) because glycogen utilization was not monitored in this present study.

The pH scale for assessment of freshness of bivalve shellfish such as oyster proposed by Pottinger (1948) needs to be re-evaluated if it is used for live mussels. It was proposed that good shucked oysters (fresh) have pH 5.9 – 6.2, off oysters 5.8, pH 5.5 – 5.7 for musty oysters while putrid oysters have pH ≤ 5.2. However, no such study has been carried out on shucked mussel or live mussels therefore this scale could not be used to determine the freshness of live mussels directly. Live oysters, mussels and or other shellfish need to be compared to verify this claim. Aside from the storage method, temperature and the type of shellfish (shucked or live), the type of equipment and method of pH determination could affect values of pH. For example, Aaraas et al. (2004) stated that the pH of ice-stored live oyster ranged between 5.6 and 6.3 using a probe specifically designed for semi-solid samples that was pressed directly into the adductor muscle unlike measuring the pH of homogenised sample which gave pH values between 6.47 – 7.01.

Nitrogenous waste comprising of ammonia ($NH_3$) and ammonium ($NH_4^+$) otherwise called total ammonia nitrogen (TAN) due to breakdown of protein is common in aquatic animals (Thomsen et al., 2016). Increasing temperature to 15 °C resulted in increased ammonia secretion in mussels because it resulted in the increase in metabolic activities (Bernardez and Pastoriza, 2013). Ammonia is used to monitor water quality as it can affect the health of aquatic animals (Zaibel et al., 2016) and a high level of ammonia is toxic to seafood. No study

on ammonia excretion in MAP live mussels has been reported in the literature. However, the results of this current study showed that ammonia (TAN) is being produced and increases with storage until the complete mortality of the mussels or end of shelf-life which could be due to increase in ammonia-oxidizing bacteria.

The result of TVC (log CFU) obtained in the second phase of this study from day 7 to day 15 in both mussel meat (6.09 – 8.13) and pouch water (6.05 – 8.82) was significantly higher (Figure 2.13) than those obtained in mussel meat (5.44 – 7.62) and pouch water (5.34 – 5.89) of the first phase (Figures 2.7 and 2.8). This could be because mussels used in the first phase were kept in recirculating aquaculture system (RAS) for a week compared to those used in this second phase, which were freshly harvested, cleaned and depurated for just 4 hr before packing. It is however not commercially viable to keep live mussels for 7 days post-harvest to reduce microbial load and gut contents. Increased TVC was observed in both pouch water and mussel meat across all the treatments from day 7 to 15. Objectionable smell was also observed in both pouch water and mussel meat of M60 and commercial packs samples on day 7, but not until day 10 for M100 and M80. Mussel degradation enhances microbial metabolism thereby increasing growth rates and exacerbating competition between viable mussels and bacteria. Bernardez and Pastoriza (2013) attributed an increased TVC in live mussels to organic waste from decomposed mussels.

The MCI of the mussels used in this study was not significantly different ($p > 0.05$) throughout the storage period except on day 15 when it dropped below marketable meat yield (35%). The initial MCI was above market index for quality mussels and remained so until it dropped on day 15. Similarly, the physical appearance of cooked mussel meat showed that samples M100

and M80 were acceptable until day 10 while M60 and commercial packs samples were acceptable until only day 7. MCI was not reported in previous studies on MAP live mussels by Pastoriza et al. (2004); Bernardez and Pastoriza (2011) nor Bernardez and Pastoriza (2013). Beyond day 7, the texture of cooked mussel meat in M60 and commercial packs became very soft when rubbed between fingers, which indicates mussel degradation. Both M100 and M80 treatments retained the freshness and quality of live mussels. TAN was observed in M80 to be below tolerance limit. Therefore, M80 appears to be the optimal initial headspace oxygen proportion (based on residual oxygen at the shelf-life and lower TAN) and so was used in the subsequent studies.

### 2.6.3 Optimisation of depuration practice of live mussels

Mussels used in phase 2 were depurated for 4 hr after harvesting. Could the short depuration time be the cause of increased microbial loads and smell due to presence of debris and microbes? In some studies, depuration has been carried out for 48 hr or more (Barile et al., 2009; Cusson et al., 2005; Polo et al., 2014). However, this is not commercially viable due to cost. What is the most suitable and cost-effective depuration duration? The current practice of the supplier of mussels is to depurate at 4°C for 1 - 4 hr. Mussels were experimentally depurated at 4°C for 4, 8 and 12 hr (Figure 2.1 and 2.2) before packing with 80% initial headspace oxygen based on the results obtained in phase 2. Commercial samples were used as a commercial pack. Filter mesh was used to collect faeces during depuration. The longer the depuration time, the more effective the depuration as seen by the increase in faecal matter on the filters (Figure 2.17).

The pH of pouch water was also monitored in this phase of the study. Similar trends were observed in both the pH of pouch waster and mussel meat. However, the pH of pouch water was higher (> 6) especially on day 7 in all the treatments. The observed increase in the pH could be due to production and release of volatile bases such as ammonia, and trimethylamine (TMA) by spoilage bacteria. Studies have shown that the pH (> 6) within and around seafood favours growth of pH sensitive Gram-negative spoilage bacteria such was *Shewanella*, *Pseudomonas* and *Photobacterium* (Fuentes-Amaya et al., 2016). No TAN in other treatments aside commercial packs was recorded on day 0 in both phases 2 and 3 in this study. This indicates that the duration of packing, number of mussels in the pack and the condition of the mussels determines production of TAN. TAN was not observed on day 15 in all the treatments because the pouch water became a slurry due to death of mussels, thereby making it difficult to measure the TAN.

One of the major purposes of depuration was to reduce microbial load of seafood especially those accumulated by filter feeders such as mussels. High initial microbial loads could result in early spoilage of seafood. Live mussels have been reported to have strong innate immune systems against bacterial invasion (Gauthier-Clerc et al., 2013; Gerdol and Venier, 2015; Martins et al., 2014). There was no significant difference in the TVC observed in samples depurated for both 8 and 12 hr on days 0, 4 and 7. The pouch water of the commercial pack was at the microbiological limit from day 0 and then raise further until it was 2 orders of magnitude above the limit on day 7. This suggested that short duration of depuration times used in the commercial product leave a lot of transient bacteria in/on the mussels and enable more substantial growth. The TVC in all treatments aside from the commercial packs was

below or at the microbial limit on day 10. While the TVC of mussel meat increased in other treatments, it was still below the unacceptable microbial limit on day 15 in the 8 hr treatment. Unlike phase 2, objectionable smell was only observed in commercial packs samples from day 7 but was not observed in the other treatments probably due to the lower TVC. The results of this study showed that both 8 and 12 hr depuration could be effective in reducing microbial load, however, due to cost, 8 hr will be recommended and used in further studies. Physical characteristics of raw and cooked mussel meat showed that mussels depurated for 8 and 12 hr were still acceptable until day 10 while 4 hr depurated, and commercial packs samples were only acceptable until day 7.

## 2.7 Conclusion

In the first phase of this study, storage of MAP live mussels at 4°C was more effective in terms of shelf-life extension compared to 6.5°C – 13°C. The results of the second phase of this study showed that high headspace oxygen appeared to result in increased metabolic activities among the mussels and this led to higher excretion of TAN to above the tolerance limit (Barrento et al., 2013) that could lead to mortality. Packs with 80% initial headspace oxygen showed lower metabolic activity that resulted in secretion of total ammonia nitrogen (TAN) that remained below the tolerance limit. The last phase of this study showed that 8 hr depuration of harvested mussels was more practically and economically effective when compared to other treatments. To preserve the freshness and quality of MAP live mussels until consumption, harvest mussels should therefore be depurated for 8 hr, packed with 80% initial headspace oxygen (100% $O_2$ had greater TAN production and possibly high rancidity)

and stored at 4°C. There is need to investigate microbial quality, freshness and spoilage metabolites of MAP live mussels in more depth during storage. This study was limited by the number of mussels in each laboratory pack compared with > 40 mussels in the commercial packs. Similarly, the cooked mussels that were not tasted to determine the palatability because safety cannot be guarantee without extensive microbiological test, hence, this was not done.

# Chapter 3: Evaluation of microbial quality and of freshness and spoilage volatile metabolites in the headspace of modified atmosphere-packed mussels

## 3.1 Introduction

Spoilage of mussels has been attributed to any of microbial activities and autolytic enzymatic activities that limit the shelf - life of packaged shucked mussels to 8 days (Caglak et al., 2008). To extend the shelf - life of mussels, deliberate alteration of the gas composition of the samples also known as modified atmosphere packaging (MAP) and cold storage have been recommended (Bernardez and Pastoriza, 2011; Pastoriza et al., 2004). Modified atmosphere packaging of live mussels with 75 – 80% initial headspace oxygen and cold storage (2 - 3°C) used to extend shelf - life to 6 days (Bernardez and Pastoriza, 2011; Pastoriza et al., 2004).

To assess the freshness and quality of seafood, microbiological, sensorial and physicochemical methods are being used (Goncalves et al., 2015). Total viable count (TVC), specific spoilage organisms (SSO) and microbial community succession are used as microbiological quality indicators. Hydrogen potential (pH), total volatile nitrogen, comprising of ammonia and other nitrogenous compounds are used to assess the quality of seafood. Olfactory and organoleptic assessments are the two methods for sensory evaluation of seafood. However, these two methods are subjective, laborious and time-consuming. More recently, evaluation of volatile metabolites produced during storage of seafood has helped overcome these limitations (Fratini et al., 2012).

Volatile metabolites (VOC) are low molecular weight organic compounds indicating freshness or spoilage of mussels (Guen et al., 2000; Tuckey et al., 2013). They are useful in the evaluation of seafood freshness, including mussels (Aru et al., 2016a, b). This is because volatile

metabolites are the aromas and flavours that most influence consumers' acceptance or rejection (Alasalvar et al., 2005).

Previous studies on volatile metabolites of fresh and spoilt mussels (*Mytulis edulis*) have been conducted. More than 120 volatile metabolites were identified. It was concluded that esters increased significantly during storage and were therefore attributed to spoilage of mussels, while aldehydes decreased during storage and were attributed to the freshness of mussels (Yasuhara, 1987). A similar study was conducted on mussel meat stored at - 80°C with 100 volatiles identified (Yasuhara and Morita, 1987). Guen et al. (2000) identified 85 volatile metabolites from cooked extracts of wild mussels (*M. edulis*) stored at - 20°C while 40 volatiles were detected from cooked mussels stored at 4°C. Aru et al. (2016b), also studied volatile metabolites produced from freshly harvested mussels (*M. galloprovincialis*) from different locations and stored at 4°C for 24 hours.

Fratini et al. (2012) investigated volatile metabolites of live mussels purchased from a local market. Mussels were only kept on ice after purchase and during processing. It was concluded that degradation of polyunsaturated fatty acid (PUFA) contributed to spoilage volatile metabolites. Tuckey et al. (2013) investigated volatile metabolites in Greenshell$^{TM}$ mussels (*Pernia canaliculus*) stored at approximately 6°C for 8 days. They observed that dimethyl sulphide, 1-penten-3-ol, 1-hexen-3-ol and 1-octen-3-ol increased during storage, indicating spoilage volatile metabolites whereas, pentanal, hexanal, heptanal, octanal and 3-undecen-2-one decreased thereby indicating freshness volatile metabolites. In all these studies, it was not stated if the mussels were depurated or not. Similarly, the microbial quality of the mussels

was not investigated. None of the studies involved modified atmosphere packaging of live mussels.

Since volatile metabolites significantly determine perceptions of freshness and or spoilage of seafood, the use of an appropriate method that is sensitive, robust and efficient is important (Fratini et al., 2012). The method used for studying volatile metabolites of seafood could influence the type of the volatile metabolites detected and identified (Zhang et al., 2009). Different methods such as dynamic headspace analysis, steam distillation headspace analysis -SHA (Alasalvar et al., 2005; Aro et al., 2003; Selli et al., 2006) and simultaneous distillation–extraction (Selli and Cayhan, 2009) have been used to study volatile metabolites in seafood. However, these methods are laborious, time-consuming, require large volume of organic solvents, multiple steps and large amounts of samples (Fratini et al., 2012; Selli and Cayhan, 2009; Zhang et al., 2009).

Similarly, thermal extraction and distillation steps could result in degradation of unstable volatile metabolites (Fratini et al., 2012). However, the advent of gas chromatography technology in combination with solid phase extraction has helped to overcome these challenges (Zhang et al., 2009). More recently, solid phase micro-extraction (SPME) which is simple, non-invasive, fast, sensitive, reproducible and does not involve use of extraction solvents is being used for the headspace analysis of volatile metabolites in seafood (Fratini et al., 2012; Zhang et al., 2009). It could also be used for headspace analysis (headspace-solid phase micro-extraction; HS-SPME) of samples. This could be combined with GC/MS (HS-SPME GC/MS) for results that are more robust.

Studies on modified atmosphere packaging of live mussels have been reported (Bernardez and Pastoriza, 2011, 2013; Pastoriza et al., 2004). However, no study has been carried out on the microbial quality and VOC produced during storage of modified atmosphere packaged live mussels. This present study, therefore, aimed to evaluate the microbial quality, freshness and spoilage volatile headspace metabolites of depurated and undepurated modified atmosphere packaged live for 15 days storage at 4°C. HS-SPME GC/MS was used to monitor the evolution of volatile metabolites responsible for freshness and spoilage of live mussels. It is expected that this study will provide a method for evaluating freshness and spoilage volatile metabolites of live mussels during storage, identify markers for freshness or spoilage, and provide information on the effect of depuration on freshness or spoilage of live mussels.

## 3.2 Materials and methods

### 3.2.1 Mussels collection and packaging

Samples of freshly harvested and cleaned live mussels were obtained from a commercial grower from the east coast of Tasmania and transported on ice to the laboratory in Styrofoam boxes. A data logger was used to monitor the temperature during transport. Half the mussels were depurated in three 10 L tanks for 8 hr as determined from optimization experiments reported in Chapter 2 of this thesis. The depuration followed the standard commercial procedure where 3 kg of mussels was added to 10 L of water. The remaining half were not depurated but packed immediately and stored at 4°C. After depuration, 16 – 17 mussels were weighed and placed in BT97/40 Barrier black trays (7 x 40 mm; Alto Packaging Ltd, Australia), 50 mL of sterile fresh water was then added to mimic commercial practice. Trays with mussels were transferred to a semi-automatic modified atmosphere packaging machine (Multivac T100, Germany) equipped with a vacuum pump for air flushing. Trays were filled with 80% $O_2$ and 20% $N_2$ initial headspace gases (food grade Oxygen, BOC, Australia) and the trays sealed using a sealing temperature of 140 - 142˚C. All trays were stored 4°C for 15 days post packing and were used for the experiments in Chapter 3 and 4. Triplicate samples of depurated (500 g), undepurated (500 g) and commercially MAP-packaged (1 kg) mussels used as reference were analysed on days 0, 4, 7, 10 and 15 for headspace gases, mortality percentage, microbial load, physico-chemical analysis, and meat condition index.

### 3.3.2 Headspace oxygen analysis of MAP live mussels

Prior to opening the samples, the headspace oxygen and $CO_2$ levels were analysed using a gas analyser machine (PBI Dansensor CheckPoint II, Denmark) as described in Chapter 2 (section 2.2.1.6). Briefly, a rubber septum was placed on each pack and an extraction needle was inserted after initial calibration with ambient air (Milne and Powell, 2014). Obtained gas levels were reported as percentages (%). The remaining analyses were conducted upon opening each pack.

### 3.2.3 Mortality

Mussels were checked for mortality by gently tapping mussels that were opened. Mortality was evaluated following the method of Goncalves et al. (2009) and reported as: Mortality (%) = (dead mussels) / (total mussels) x 100.

Each pack was also observed for mussels with any indication of broken shells. A threshold of 20% acceptable mortality was used (Bernardez and Pastoriza, 2011).

### 3.2.4 Microbiological analysis

Microbiological analysis was carried as follows: On each sampling time (day), 70% ethanol was used to clean the surface of the packs before opening with sterile scissors. Thereafter, 15 g of mussel meat added to 135 mL of sterile 0.1% bacteriological peptone water prepared from bacteriological peptone powder (LP003T, Oxoid UK) and NaCl 5% w/v (Fisher chemicals USA)

(Dabade et al., 2015). Samples were homogenised for 60 seconds before decimally diluting 1 mL of each sample in sterile 0.85% w/v saline water. Thereafter, 10 µL from each dilution was spread on Standard Plate Count Agar (CM 0463, Oxoid UK) for enumeration of total viable count (TVC) (Powell et al., 2015) and plates were incubated as described in Chapter 2.

### 3.2.5 Physico-chemical analysis

According to Barrento et al. (2014) live mussels excrete ammonia as by-product, hence, the total ammonia nitrogen (TAN mg $L^{-1}$) was investigated using a commercial kit following manufacturer's instruction (Mars Fishcare, North America) and 4 mg $L^{-1}$ was used as tolerance limit (Barrento et al., 2014). Homogenates (50 mL) from both mussel meat and pouch water samples were collected and used for pH analysis using a calibrated pH meter (ATI Orion Research Model 250A, USA) at 25˚C.

### 3.2.6 Meat condition index (MCI)

The meat condition index which is used to indicate nutritional and quality of bivalve Orban et al. (2002) of each sample was carried out by randomly selecting 5 – 7 mussels from each pack. The mussels were weighed and then cooked for 3 minutes at 1500W in a microwave oven (Palsonic, Australia). The cooked mussel was reweighed; shell removed and the meat reweighed. The meat condition index (MCI) was analysed based on the amount of water loss after cooking in relation to the dry weight of mussels (Irisarri et al., 2015; Orban et al., 2002). As practised by the commercial supplier of mussels used in this study, a meat condition index of above 30% was used as a measure of marketability meat yield.

### 3.2.7 Volatile organic compound analysis

A multicomponent SPME fiber (50/30µm Divinylbenzene DVB)/Carboxen (CAR)/Polydimethylsiloxane Stable flex fiber) was used as it can detect a wide range of volatile types and previously trialled by the technologist in charge of the GCMS machine. The fiber was pre-conditioned by exposure to the headspace of 10 mL of water in a screwed cap vial for 20 minutes. The water sample (blank) was initially pre-incubated at 80°C for 30 minutes. Thereafter, the fiber was inserted into the injection port of GC/MS (Varian CP-3800 Gas Chromatograph) that was connected to a Bruker 300-MS TQ Mass Spectrometer for 5 minutes while the GC program was allowed to run to completion. This was carried out to ensure no impurities that could interfere with the compounds in the samples were present (Fratini et al., 2012). Homogenised samples of mussel meat or pouch water from the packs at each sampling point were used for volatile organic compound analysis. Twenty millilitres of the sample in a 40 mL septum vial was heated at 80°C for 30 min in a GC oven (Hewlett Packard 5890 series 2) to attain equilibrium. Thereafter, the pre-conditioned fibre described above was inserted into the septum at room temperature for 20 minutes to capture headspace volatiles. The exposed fiber was then inserted into the GC/MS injector for thermal desorption of volatiles for 8 minutes.

### 3.2.8 GC/MS setting

GC/MS analysis was performed using a Bruker 300-MS TQ Mass Spectrometer with a split/splitless injector mode (ratio 2:1). Compound separation was carried out using an Agilent DB-5MS capillary column 30 m length x 0.25 mm ID x 0.25 mm film thickness (Agilent

Scientific, USA) based on the following conditions: carrier gas - helium with a flow rate of 1 mL $min^{-1}$, injector temperature 270°C, initial oven temperature 40°C, held for 4 min, to 80°C at 6°C $min^{-1}$, to 250°C at 8°C $min^{-1}$ and to 290°C at 25°C $min^{-1}$. The following parameters were used for the mass detector: ion mass/charge ratio, 35 – 350 m/z, full scan mode. The source temperature of electron impact ionisation (EI) used was 220°C, line voltage of 245V and ion gauge emission current 1.00 ma.

### 3.2.9 Identification of compounds

Mass Spectrometer (MS) Data Review software version 7 (Thermofisher Scientific, USA) was used to plot and process chromatogram and spectral data obtained. The mass spectrum of each compound obtained was matched with the National Institute of Standards and Technology (NIST, USA) library of compounds using > 90% relative match as the standard. Each chromatogram was integrated by filtering the peak at 5 smooth points and the integration window was set at 0.150 peak width (sec ½HZ). The peak size rejection was set at 5000 counts.

### 3.2.10 Statistical analysis

Significant differences were tested at $p < 0.05$ based on analysis of variance (ANOVA), Tukey's post-hoc multiple comparison tests (Statistical Package for the Social Sciences SPSS version 24 – IBM) and multiple regression (Excel spreadsheet 2018). Microbiological data were expressed as the log of colony-forming units (CFU) per gram or millilitre (log CFU $g^{-1}$ or log CFU $mL^{-1}$). The microbial limit was set at 6 log CFU $g^{-1}$ or $mL^{-1}$ as recommended by (National Advisory

Committee on Microbiological Criteria for Foods, 1992). Volatile organic compound profiles obtained, including peak areas of each compound, were statistically analysed using multivariate exploratory analysis comprising of principal component analysis (PCA), partial least squares regression (PLS), and heat-map analysis (Kuuliala et al., 2018). PLS was used to identify freshness and spoilage volatiles markers based on variable importance projection (VIP) > 1 while PCA biplot and heat map were used to discriminate freshness and spoilage volatile markers (Kuuliala et al., 2018; Zaragozá et al., 2014). XLSTAT Version 2017 (XLSTAT Software, USA) was used for multivariate analyses. GraphPad Prism version 7 (GraphPad Software, CA 92037 USA) was used for all other statistical analysis.

## 3.3 Results

Two treatments of mussels were used in this study namely, depurated, undepurated and commercial packs (CP). All samples were packed at 80% initial headspace oxygen and stored at 4°C for 15 days.

### 3.3.1 Headspace oxygen analysis

There was no significant difference ($p > 0.05$) in the initial headspace oxygen in all the treatments on day 0 (78.5 – 81.6%) and day 4 (60.9 – 76.3%) but was on day 15 (Figure 3.1).

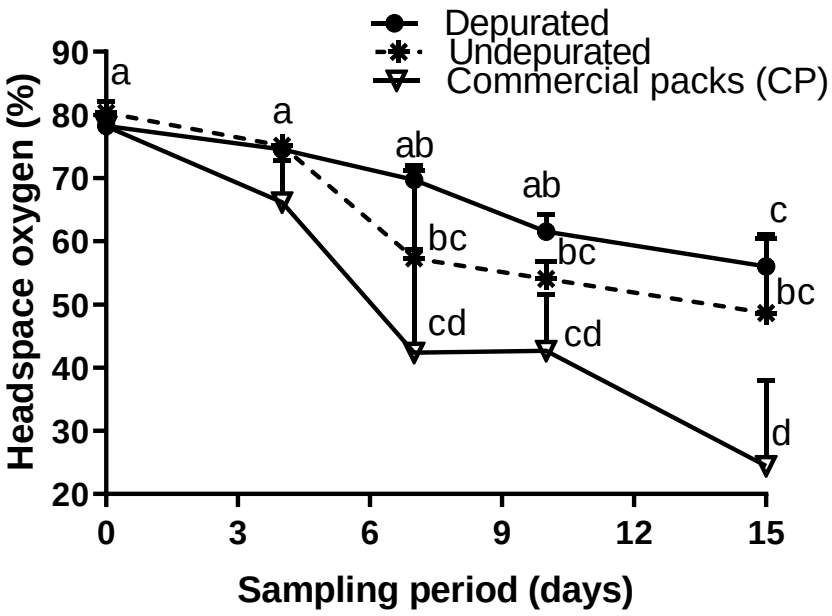


**Figure 3.1:** Headspace oxygen in depurated, undepurated and commercial packs (CP) stored at 4˚C for 15 days. Results represent the mean and standard deviation of triplicate analysis (n=3). Different superscript denotes significant difference ($p < 0.05$) in the means of headspace oxygen.

The data obtained for headspace oxygen, $CO_2$, pH, TAN, TVC and MCI in Chapter 2 and 3 were compared. However, only data obtained from samples packed with 80% initial headspace oxygen and depurated for 8 hr (based on optimization result in Phase 2) were compared and reported below. When statistically compared with the data obtained from the different experiments and times reported in Phases 2 and 3, there were no significant differences between the headspace oxygen in depurated (Figure 3.1), M80 (Figure 2.9) and 8 hr depuration (Figure 2.18).

There was no significant difference ($p > 0.05$) in the headspace oxygen of depurated and undepurated treatments on day 7 (41.2 – 71.5%), on day 10 (50.9 – 64.5%) and on day 15 (41 – 62.3%). However, individual treatments and the commercial packs do show a significant decrease with time, with the commercial packs being the lowest. No significant difference ($p > 0.05$) in the headspace oxygen of undepurated and commercial packs on day 7 (24.9 – 66.8% and day 10 (35.8 – 55.9%). The CP samples had a mean headspace oxygen significantly lower than that of depurated samples from day 7. By the commercial shelf-life day (day 10), no significant difference ($p > 0.05$) was observed between day 0 and 10 ($p=0.14$) in depurated samples, however, a significant difference ($p < 0.05$) was observed between day 0 and 10 ($p=0.01$) in undepurated and commercial packs ($p=0.0006$).

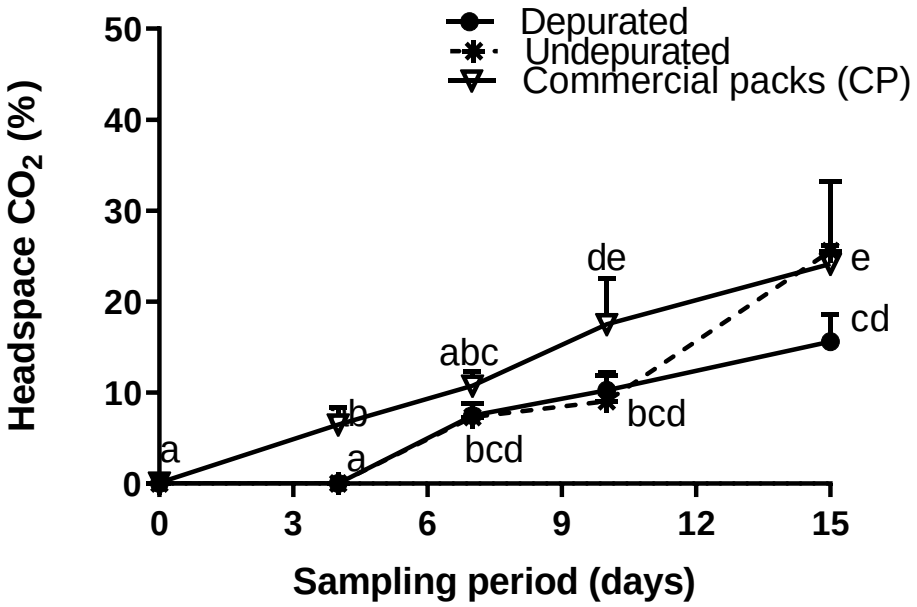


**Figure 3.2**: Headspace $CO_2$ in depurated, undepurated and commercial packs (CP) stored at 4˚C stored for 15 days. Results represent the mean and standard deviation of triplicate (n=3) analysis. Different superscript denotes significant difference ($p < 0.05$) in the means of $CO_2$.

The $CO_2$ was below the detection limit < 0.3% on day 0 and 4 in depurated and undepurated treatments while the commercial pack was 0.3% on day 0 and 6.5 ± 1.9% on day 4 (Figure 3.2). A significant increase in $CO_2$ (6 – 12.6%) was observed in both treatments on day 7 and a further increase to a mean value of 25% $CO_2$ was seen in the undepurated treatment on day 15. $CO_2$ in the commercial packs was significantly higher (24%) on day 15 compared to 11% on day 7. However, there were no significant differences ($p > 0.05$) between the treatments and commercial packs on days 7 and 10. By day 15, the commercial packs had significantly more $CO_2$ than did the depurated, but not the undepurated packs.

In comparison with the data obtained in Chapter 2 (Phases 2 and 3), there was no significant difference ($p > 0.05$) in the headspace $CO_2$ between the depurated (Figure 3.2) and 8 hr (Fig 2.19) treatments but there were significant differences ($p < 0.05$) between M80 (Figure 2.10)

and other treatments (depurated and 8 hr depuration). The headspace $CO_2$ in M80 was significantly higher on days 10 (21%) and 15 (30%) unlike 8 hr depuration (9% and 12%) and depurated (10% and 16 %).

### 3.3.2 Mortality

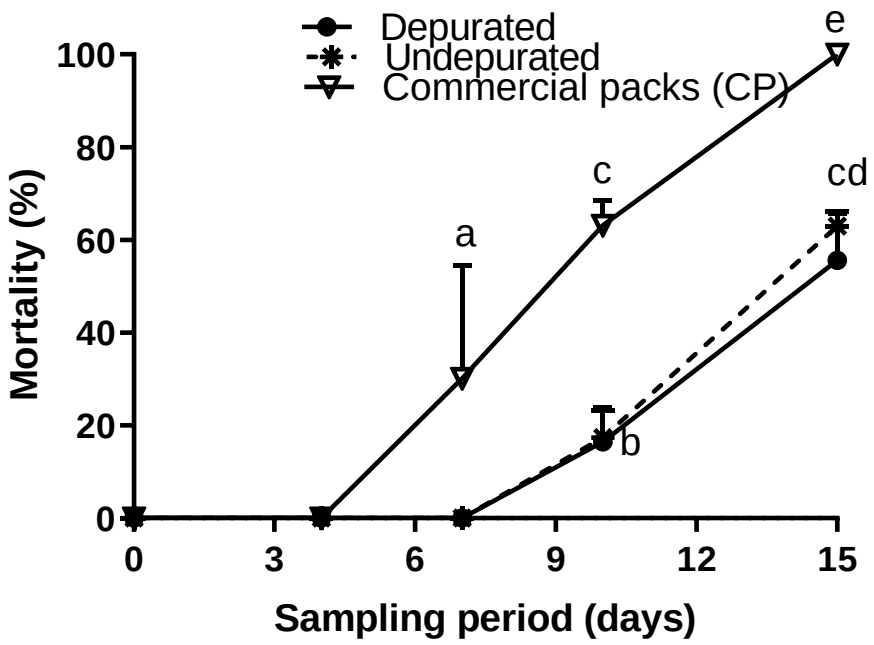


**Figure 3.3**: Mortality (%) of depurated, undepurated and commercial packs (CP) stored at 4˚C stored for 15 days. Results represent the mean and standard deviation of triplicate (n=3) samples. The broken line represents the mortality threshold (20%). Different superscript denotes significant difference ($p < 0.05$) in the means of mortality.

Mortality was expressed as a percentage of total mussels in each pack (Figure 3.3). No mortality was detected in any treatment on days 0 and 4 but was detected in the commercial packs on day 7 (30.14%). On day 10, a significant increase in mortality was observed in the commercial packs (63.17%) while the first mortality was detected in depurated (16.42%) and undepurated (17.37%) treatments. On day 15, a significant increase in mortality was observed in depurated (55.56%), undepurated (62.96%) and commercial packs (100%).

### 3.3.3 Physicochemical analysis

#### 3.3.3.1 pH

No significant difference ($p > 0.05$) was observed in the pH of pouch water added to undepurated and in the commercial packs on day 0 (6.12 – 6.32) (Fig. 3.4). However, the pH of the pouch water added to the depurated samples (6.50) was significantly higher than the pouch water in the undepurated treatment.

A similar pattern was observed on day 4 when the mean pH of the depurated treatment was the highest recorded in the experiment. There was no significant difference ($p > 0.05$) in the pH of both treatments and the commercial packs on day 7 (6.29 – 6.43). While the pH of commercial packs was significantly lower on day 10 (6.07) compared to the undepurated treatment, there was no further significant difference ($p > 0.05$) in either treatment (depurated: 6.34 and undepurated: 6.42) or the commercial packs (6.07).

When compared with the data obtained in Chapter 2 (Phases 2 and 3), there was no significant difference ($p > 0.05$) in the pH of pouch water between M80 and 8 hr depuration but there were significant differences ($p < 0.05$) between depurated mussels (Figure 3.4), 8 hr depuration (Fig 2.20A) and M80 (Figure 2.11A). The pH of depurated mussels was significantly higher ($p < 0.05$) on days 0 (pH = 6.5) and 4 (pH = 6.8) unlike 8 hr depuration (pH = 5.8 and 6.2) and M80 (pH = 6 and 6.2). However, no significant differences were observed on other storage days.

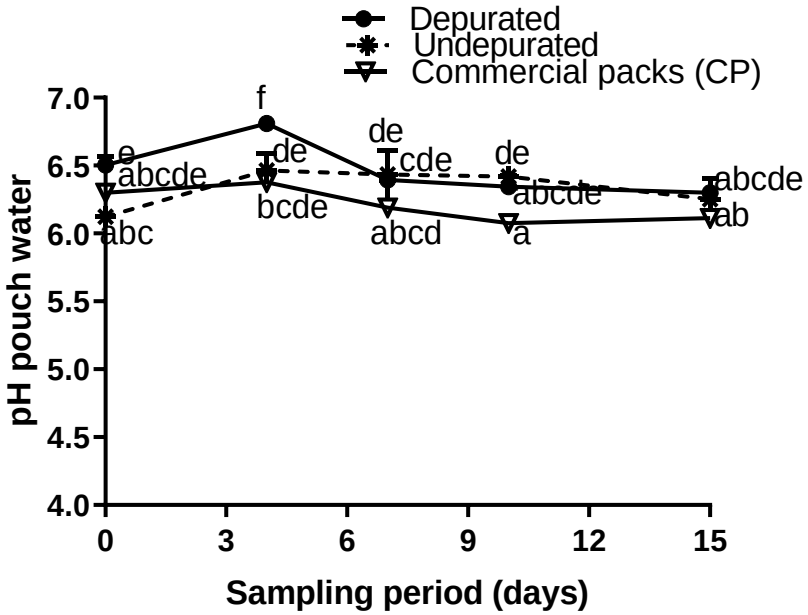


**Figure 3.4**: pH of pouch water of depurated, undepurated and commercial packs (CP) stored at 4˚C stored for 15 days. Results represent the mean and standard deviation of triplicate (n=3) samples. Different superscript denotes significant difference ($p < 0.05$) in the means of pH.

There were no significant differences ($p > 0.05$) between the pH of mussel meat in the treatments and commercial packs. However, the mean pH of mussel meat of commercial packs was significantly lower on day 0 (pH = 4.8) than day 15 (pH = 5.9) (Figure 3.5). When compared with the data obtained in Chapter 2 (Phases 2 and 3), there were no significant differences ($p > 0.05$) in the pH of mussel meat between the depurated mussels (Figure 3.4), M80 (Figure 2.11B) and 8 hr depuration (Figure 2.20B).

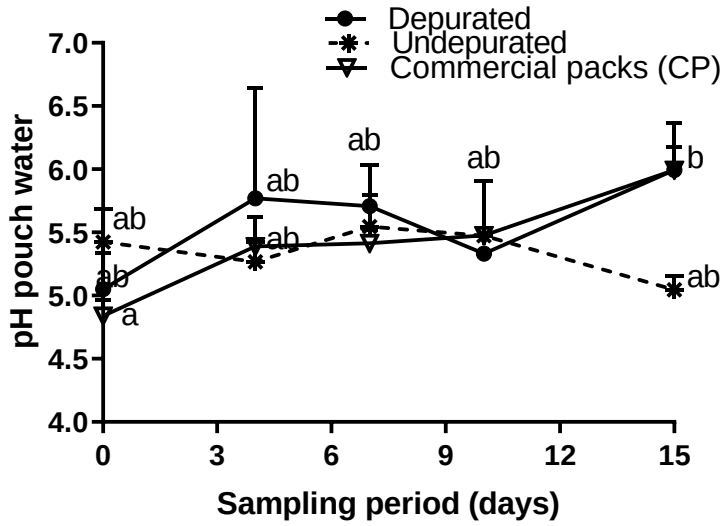


**Figure 3.5**: pH of mussel meat of depurated, undepurated and commercial packs (CP) stored at 4˚C stored for 15 days. Results represent the mean and standard deviation of triplicate (n=3) samples. Different superscript denotes significant difference ($p < 0.05$) in the means of pH.

### 3.3.3.2 Total ammonia nitrogen -TAN (mg $mL^{-1}$)

The mean TAN was below detection (< 0.5 mg $mL^{-1}$) on day 0 in all the treatments and commercial packs (Figure 3.6). It was however detected on day 4 in all the treatments without significant differences ($p < 0.05$) between undepurated (3.67 mg $mL^{-1}$) versus depurated mussels (1.83 mg $mL^{-1}$) and the commercial packs at 1.83 mg $mL^{-1}$, with all concentrations below the tolerance limit (5 mg $mL^{-1}$). On day 7 and 10, the mean concentration of TAN in undepurated mussels was above the tolerance limit of 5 mg $mL^{-1}$.

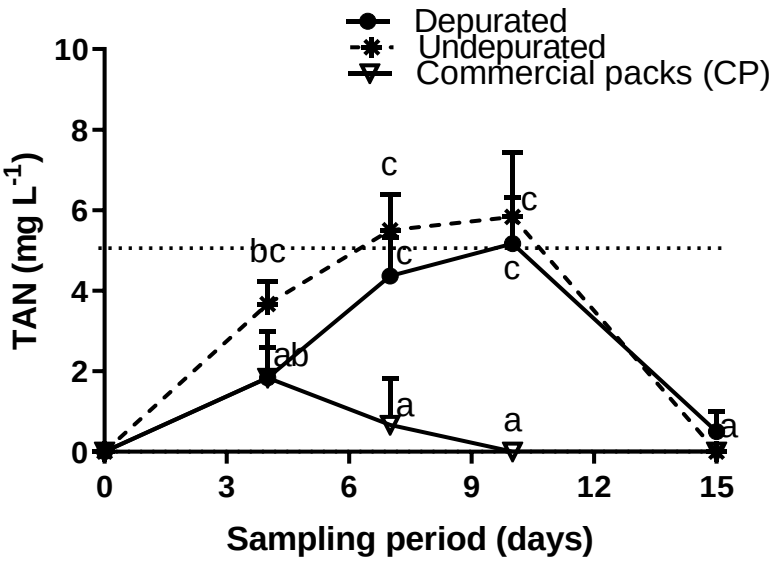


**Figure 3.6**: Total ammonia nitrogen (TAN mg $L^{-1}$) of depurated, undepurated and commercial packs (CP) stored at 4˚C stored for 15 days. Results represent the mean and standard deviation of triplicate (n=3) samples. The broken line represents mussels' tolerance limit for ammonia. Different superscript denotes significant difference ($p < 0.05$) in the means of TAN.

On day 15, the mean TAN in undepurated mussels was below detection while the mean TAN in depurated mussels was significantly lower (0.5 mg $mL^{-1}$) than it was at day 10. TAN was not detected in commercial packs on day 15. TAN was not detected in commercial packs on day 15. Overall, there were significant differences ($p < 0.05$) between commercial packs versus undepurated and commercial packs versus depurated mussels. In comparison with the data obtained in Chapter 2 (Phases 2 and 3), there was no significant difference ($p > 0.05$) in the TAN between depurated mussels (Figure 3.6, Chapter 3), M80 (Figure 2.12, Chapter 2 – phase 2) and 8 hr depuration (Fig 2.21, Chapter 2 – phase 3).

### 3.3.4 Microbiological analysis

The mean TVC of pouch water from the undepurated treatment (6.42 log CFU $mL^{-1}$) and from the commercial packs (6.67 log CFU $mL^{-1}$) were not significantly different ($p > 0.05$) from each

other and were above acceptable microbial limit on day 0 and subsequently until the end of the experiment.

In comparison with the data obtained in Chapter 2 (Phase 2 and 3), there were no significant differences ($p > 0.05$) between in the TVC of pouch water of depurated mussels (Figure 3.7A), M80 (Figure 2.13A, Chapter 2) and 8 hr depuration (Fig 2.22A, Chapter 2). However, the TVC of pouch water was highest in M80 on days 7 (log 7.9 CFU $mL^{-1}$), 10 (log 7.6 CFU $mL^{-1}$) and 15 (log 8.4 CFU $mL^{-1}$) but was lowest in depurated mussels (log 6.2, 6.3 and 6.9 CFU $mL^{-1}$).

The mean total viable count (TVC) was significantly below (3.3 log CFU $g^{-1}$) acceptable microbial limit (6 log CFU $g^{-1}$) in depurated treatment on day 0 (Figure 3.7). Mean TVC in undepurated (4.79 log CFU $g^{-1}$) and commercial packs (5.20 log CFU $g^{-1}$) were not significantly different ($p > 0.05$) and were also below the acceptable microbial limit. On day 4 and 7, the mean TVC in depurated treatment significantly increased but was still below the acceptable microbial limit. The mean TVC of the undepurated and commercial packs significantly increased above the acceptable microbial limit from day 7 and subsequently. As observed in mussel meat (Figure 3.7B), the mean TVC in pouch water for the depurated treatment on day 0 was significantly below (5.17 log CFU $mL^{-1}$) the acceptable microbial limit (6 log CFU $mL^{-1}$).

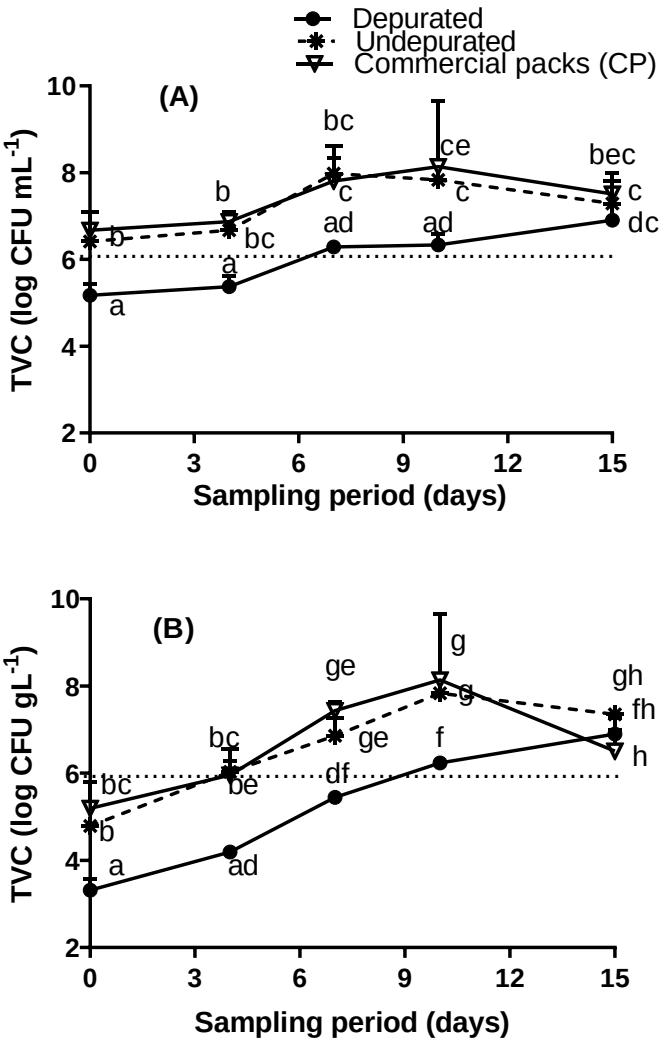


**Figure 3.7**: Total viable count (TVC) of pouch water (A) and mussel meat (B) of depurated, undepurated and commercial packs (CP) of stored at 4˚C stored for 15 days. Results represent the mean and standard deviation of triplicate (n=3) samples. The broken line represents acceptable microbial limit. The start of objectionable smell was on day 7 in undepurated mussels and commercial packs but day 10 in depurated mussels. Different superscript denotes significant difference ($p < 0.05$) in the means of TVC.

When compared with the data obtained in Chapter 2 (Phase 2 and 3), there was no significant difference ($p > 0.05$) in the TVC of mussel meat between the depurated mussels (Figure 3.7B, Chapter 3) and 8 hr depuration (Fig 2.22B, Chapter 2) but the TVC of depurated M80 (Figure 2.13B, Chapter 2) was significantly higher ($p < 0.05$) than depurated mussels. However, there was no significant difference ($p > 0.05$) between M80 and 8 hr depuration.

### 3.3.5 Meat condition index

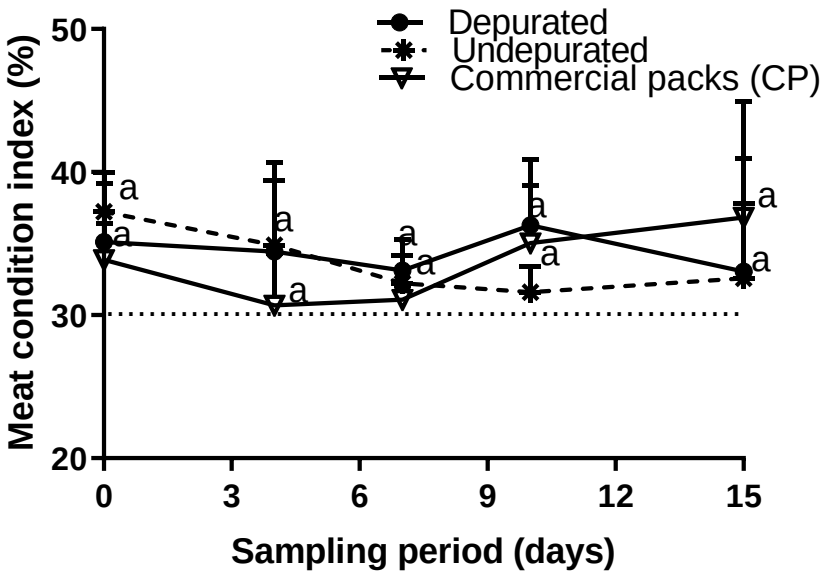


**Figure 3.8**: Meat condition index of depurated, undepurated and commercial packs (CP) samples stored at 4˚C stored for 15 days. Results represent the mean and standard deviation of triplicate (n=3) samples. The broken line represents premium meat yield and meat condition.

There was no significant difference ($p > 0.05$) in the MCI of mussel meat between the treatments during storage (Figure 3.8). The mean MCI of all the treatments was above marketable meat yield (30%). In comparison with the data obtained in Chapter 2 (Phase 2 and 3), there was no significant difference ($p > 0.05$) in the MCI between depurated mussels (Figure 3.8), M80 (Figure 2.14) and 8 hr depuration (Fig 2.23).

## 3.4 Evolution of volatile metabolites

A total of 65 volatile metabolites comprising aldehydes (13), alcohols (8), ketones (5), nitrogen compounds (2), sulphur compounds (4), esters (1), organic acids (2), furans (5), volatile

phenols (3), aromatic hydrocarbons (13) and hydrocarbons (9) were detected and identified in the headspace of mussel meat or in pouch water during 15 days' storage at 4°C.

Based on the selection criteria of Partial Least Square analysis that was used, the volatiles were reduced to 58 (Table 3.1). However, principal component analysis (PCA) reduced these further to 32 with 17 volatiles identified as freshness markers and 15 volatiles identified as spoilage markers (Tables 3.2 and 3.3). Based on normalised peak areas, the concentration of freshness volatiles decreased with storage days, whereas the concentration of spoilage volatiles increased. In the pouch water, spoilage volatiles such as dimethyl disulphide, heptadecane were common to the treatments and commercial packs. Indole, methyl phenol and tetradecanone were only present in undepurated mussels and the commercial packs. Freshness volatiles present in both undepurated and depurated mussels were nonanone and tribromethane (Tables 3.2). In the mussel meat, spoilage volatiles in the treatments and commercial packs include indole and octadienol while freshness volatiles include nonedienol, pentadecane, ethyl hexane and benzaldehyde (Table 3.3).

Principal component analysis (PCA) (Figure 3.9 – 3.14) was used to identify potential freshness and spoilage volatiles based on correlation with TVC. The results of PCA showed that volatiles present in depurated pouch water and mussel meat clustered together on day 0, 4 and 7 indicating freshness (Figure 3.9) while volatiles indicating spoilage clustered together on day 10 and 15 (Figure 3.10).

Unlike depurated treatment, the results of PCA showed that volatiles present in undepurated pouch water clustered together on day 0, 7 and 15 (Figure 3.11). While volatiles on day 4 and 10 clustered together. In mussel meat, volatiles on day 0 and 4 clustered together while days 7, 10 and 15 clustered together (Figure 3.12). In the commercial packs, the volatiles present in pouch water clustered together on day 0 and 4 indicating freshness (Figure 3.13) while volatiles indicating spoilage clustered together on day 7, 10 and 15.

The result of the PCA of mussel meat in the commercial packs (Figure 3.14) was like that of undepurated mussel meat. Heat-maps (Figure 3.15 - 3.20) were further used to clarify and ascertain potential freshness and spoilage volatiles based on correlation with TVC. As microbes increase, production of by-products and degradation of mussels increases releasing more VOC into the packs. The evolution of spoilage volatiles increased with storage days as indicated with colour change from red to green based on the heat-map. The evolution of freshness volatiles decreased with storage days as indicated with colour change from green to red based on the heat-map.

**Table 3.1:** Potential freshness and spoilage volatiles of MAP live mussels stored at 4°C for 15 days as determined by PLS regression analysis. Selection criteria (VIP > 1, positive correlation with TVC as the dependent variable (Kuuliala et al., 2018). X signified presence.

| VOC | Depurated | | Undepurated | | Commercial packs | |
|---|---|---|---|---|---|---|
| | Pouch water | Mussel meat | Pouch water | Mussel meat | Pouch water | Mussel meat |
| 2,4-Decadienal | | | | | x | |
| 2-Decanol | | | | | x | |
| 2-Ethyl-5-methylfuran | x | x | | x | | |
| 2-Ethylfuran | x | | x | x | | |
| 3,5 - Octadiene | x | x | x | x | | |
| 3-Ethylthiophene | | x | | | | |
| 3-Methylbutanol | x | | | x | | |
| Acetic acid | | x | | x | x | x |
| Benzaldehyde | x | x | x | x | | x |
| Bis (Dimethyl ethyl) Benzene | x | x | | | | x |
| Butyl ester | | | | x | | x |
| C12 - C15 aldehyde | x | x | | | | |
| Chlorobenzene | | | | x | | |
| Decanal | x | | | x | | x |
| Diethyl furan | | x | | | | x |
| Dimethyl butanal | | x | | x | | x |
| Dimethyl disulphide | x | | | | x | |
| Dimethyl methylamine | x | | | | | |
| Dimethyl sulphide | x | x | x | | | |
| Dimethyl trisulphide | x | x | | | | |

**Table 3.1:** Potential freshness and spoilage volatiles of MAP live mussels stored at 4°C for 15 days as determined by PLS regression analysis. Selection criteria (VIP > 1, positive correlation with TVC as the dependent variable (Kuuliala et al., 2018) (Continued). X signified presence.

| VOC | Depurated | | Undepurated | | Commercial packs | |
|---|---|---|---|---|---|---|
| | Pouch water | Mussel meat | Pouch water | Mussel meat | Pouch water | Mussel meat |
| Dithiomethane | | | | | x | |
| Dodecane | | x | | x | x | x |
| Ethyl benzene | x | | | | x | x |
| Ethyl hexane | | x | | | x | |
| Ethyl phenol | | x | x | | | |
| Fatty acid | x | | | | | |
| Heptadecane | x | | x | | x | x |
| Heptanol | | x | x | | x | x |
| Heptenal | | x | | x | | x |
| Hexanal | x | | x | x | | |
| Indole | | x | | x | x | x |
| Lilac aldehyde | | | x | | | x |
| Methyl heptanone | x | x | x | | x | |
| Methyl phenol | | | | x | x | |
| Naphthalene | | | | | x | |
| Nonanal | | | x | x | | |
| Nonanone | x | | x | | | x |
| Nonedienol | x | x | x | | | x |
| Octadecanal | x | | x | | x | |
| Octadiene | x | x | | x | | |

**Table 3.1:** Potential freshness and spoilage volatiles of MAP live mussels stored at 4°C for 15 days as determined by PLS regression analysis. Selection criteria (VIP > 1, positive correlation with TVC as the dependent variable (Kuuliala et al., 2018) (Continued). X signified presence.

| VOC | Depurated | | Undepurated | | Commercial packs | |
|---|---|---|---|---|---|---|
| | Pouch water | Mussel meat | Pouch water | Mussel meat | Pouch water | Mussel meat |
| Octadienol | x | x | x | x | | x |
| Octanal | | | | | x | |
| Octenal | | | | | x | |
| Octene | | | | x | | x |
| Octenol | | x | | x | | x |
| Octenone | | | x | | | x |
| Pentadecane | | x | | | | x |
| Pentylfuran1 | | | x | x | x | |
| Pentylfuran2 | x | | x | x | | |
| Phenol | | x | | | x | |
| Styrene | | | | | x | |
| Tetradecanone | | | | | x | |
| Tetramethyl pentane | x | | x | x | | x |
| Thiohexene | | | | x | | |
| Toluene | x | | | | | x |
| Tribromomethane | x | x | x | | x | |
| Tridecane | | x | | x | x | |
| Xylene | x | x | | | | x |

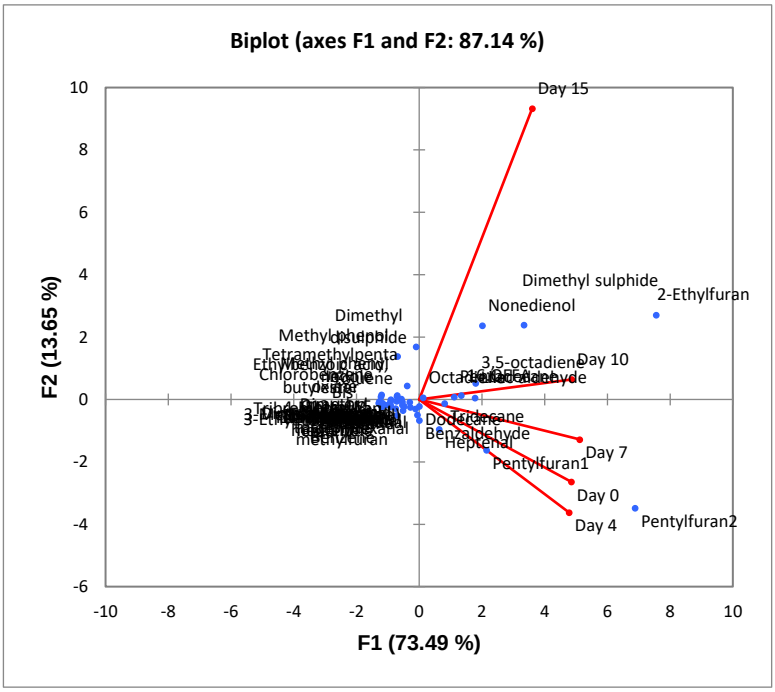


**Figure 3.9:** Biplot of the principal components analysis (PCA) of identified volatile metabolites in depurated mussels (pouch water) stored at 4°C for 15 days.

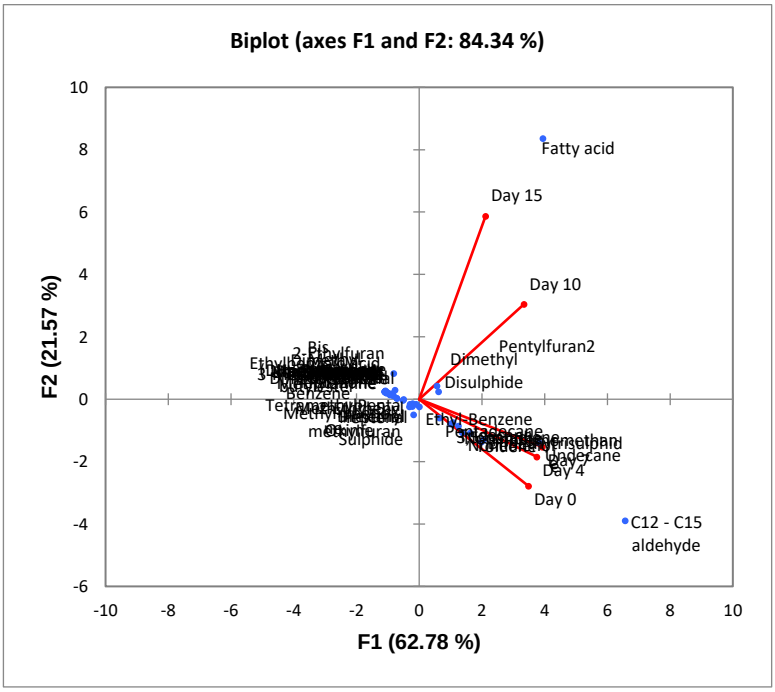


**Figure 3.10**: Biplot of the principal components analysis (PCA) of identified volatile metabolites in depurated mussels (mussel meat) stored at 4°C for 15 days.

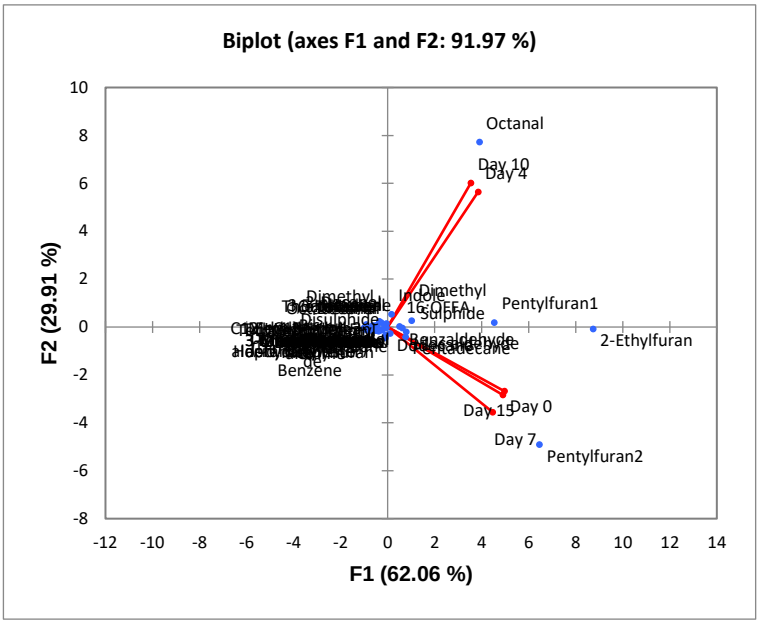


**Figure 3.11**: Biplot of the principal components analysis (PCA) of identified volatile metabolites in undepurated mussels (pouch water) stored at 4°C for 15 days.

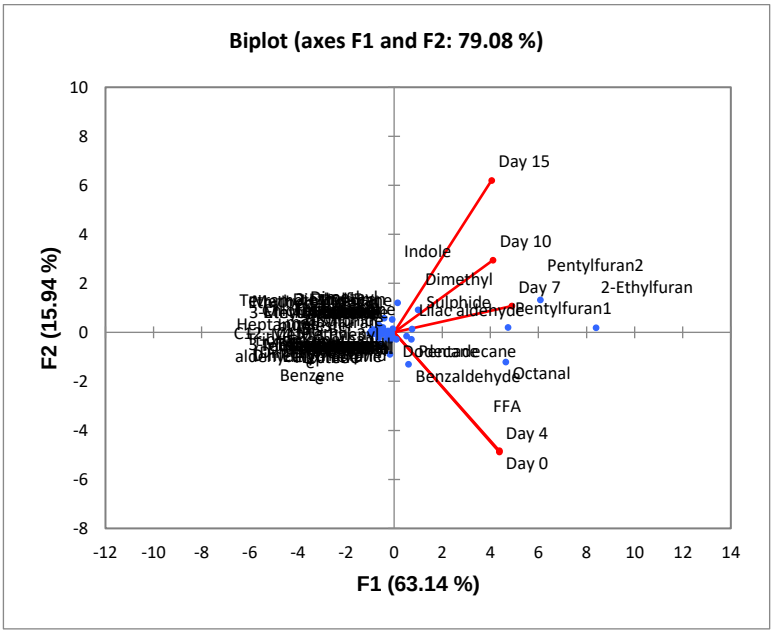


**Figure 3.12**: Biplot of the principal components analysis (PCA) of identified volatile metabolites in undepurated mussels (mussel meat) stored at 4°C for 15 days.

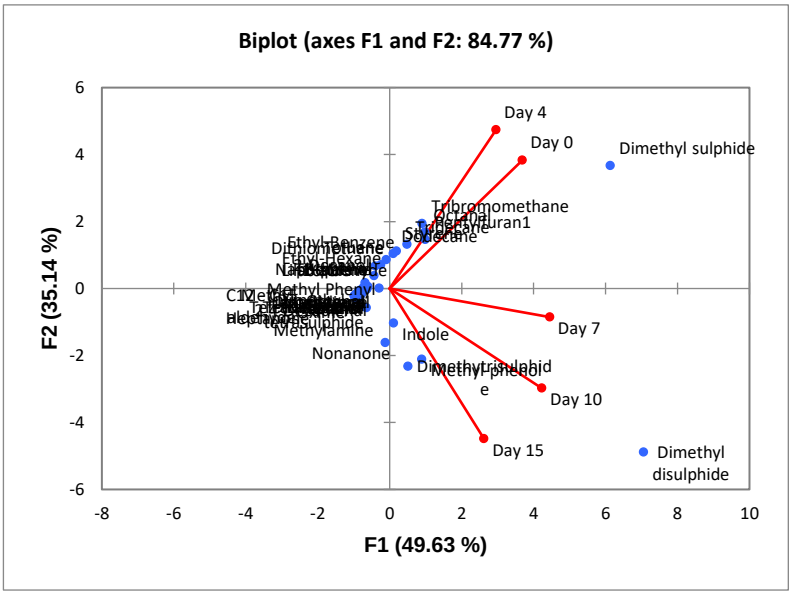


**Figure 3.13**: Biplot of the principal components analysis (PCA) of identified volatile metabolites in commercial packs (pouch water) stored at 4°C for 15 days.

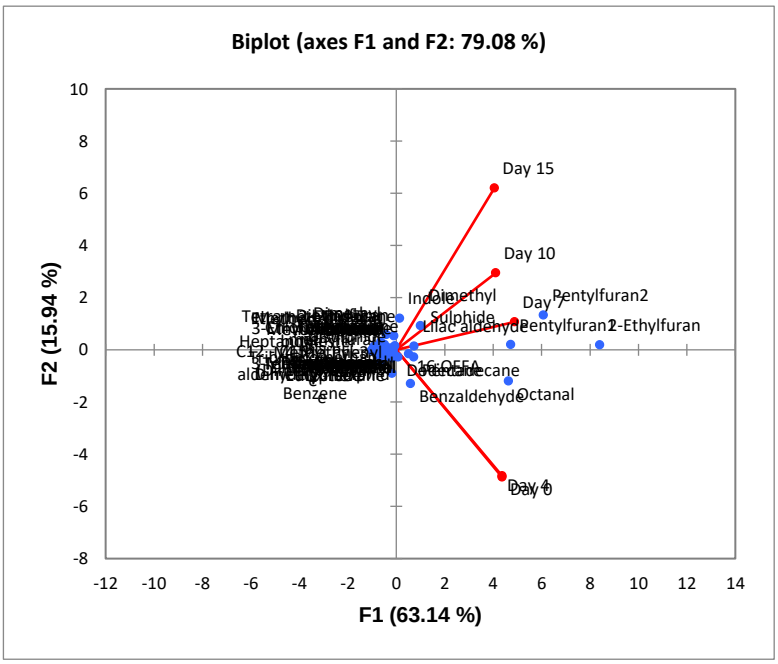


**Figure 3.14**: Biplot of the principal components analysis (PCA) of identified volatile metabolites in commercial packs (mussel meat) stored at 4°C for 15 days.

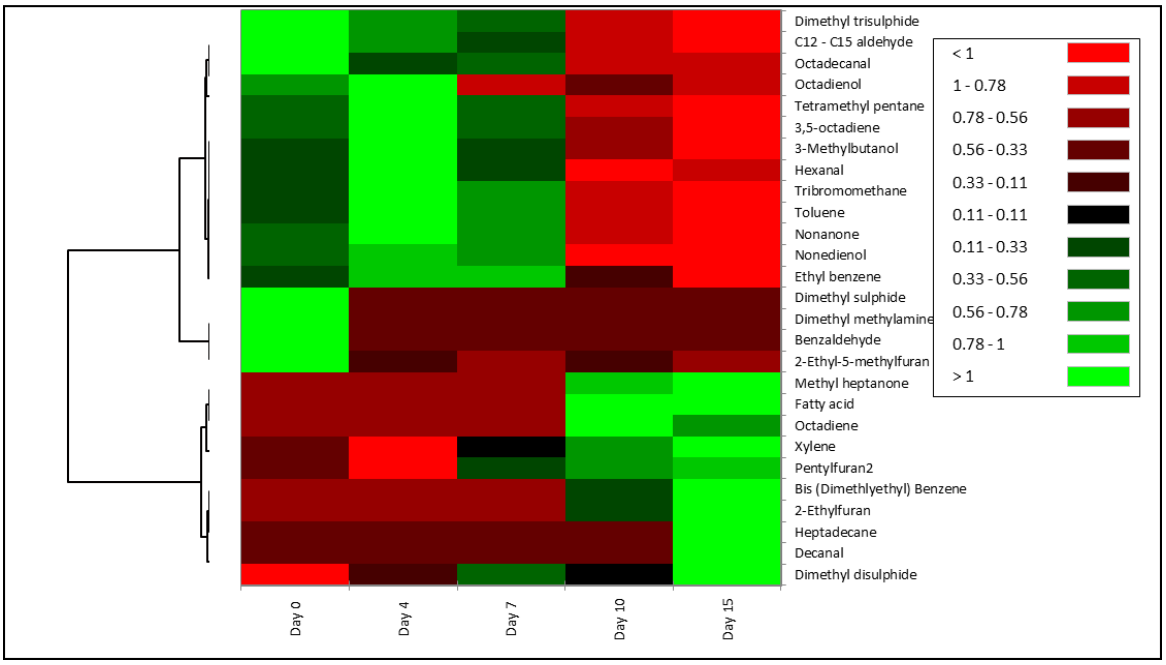


**Figure 3.15**: Heat-map of the evolution of identified volatile metabolites in depurated mussels - (pouch water) stored at 4°C for 15 days. Colour intensity indicates normalised amounts of volatiles with high concentration in red and low concentration in green.

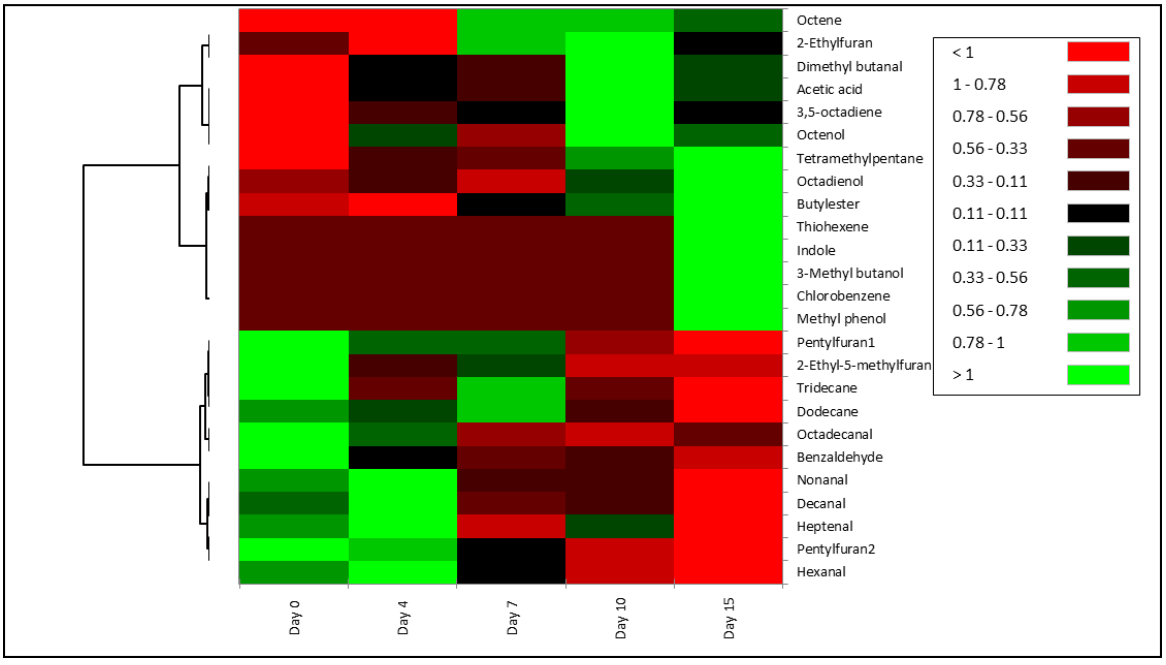


**Figure 3.16**: Heat-map of the evolution of identified volatile metabolites in depurated mussels - (mussel meat) stored at 4°C for 15 days. Colour intensity indicates normalised amounts of volatiles with high concentration in red and low concentration in green.

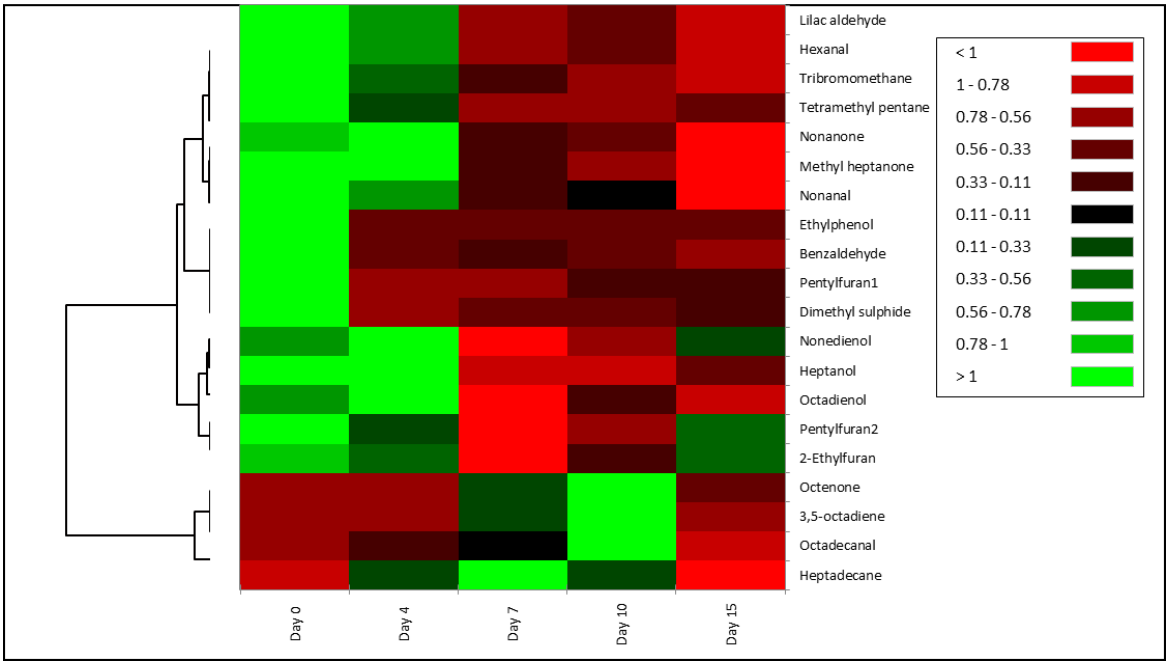


**Figure 3.17**: Heat-map of the evolution of identified volatile metabolites in undepurated mussels - (pouch water) stored at 4°C for 15 days. Colour intensity indicates normalised amounts of volatiles with high concentration in red and low concentration in green.

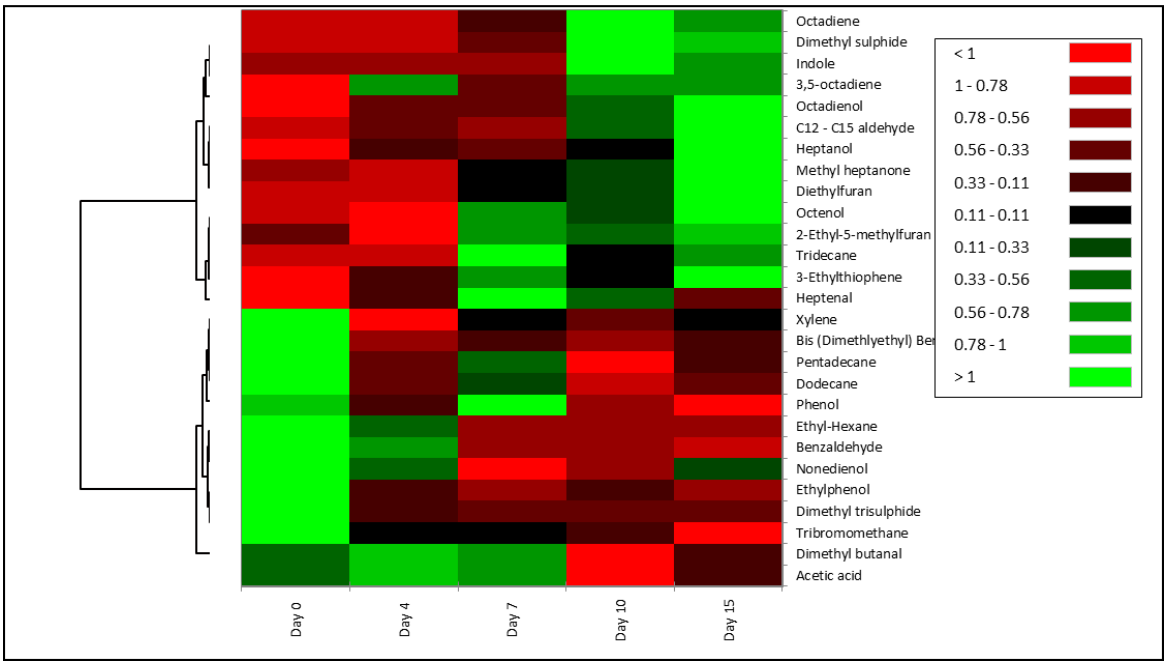


**Figure 3.18**: Heat-map of the evolution of identified volatile metabolites in undepurated mussels - (mussel meat) stored at 4°C for 15 days. Colour intensity indicates normalised amounts of volatiles with high concentration in red and low concentration in green.

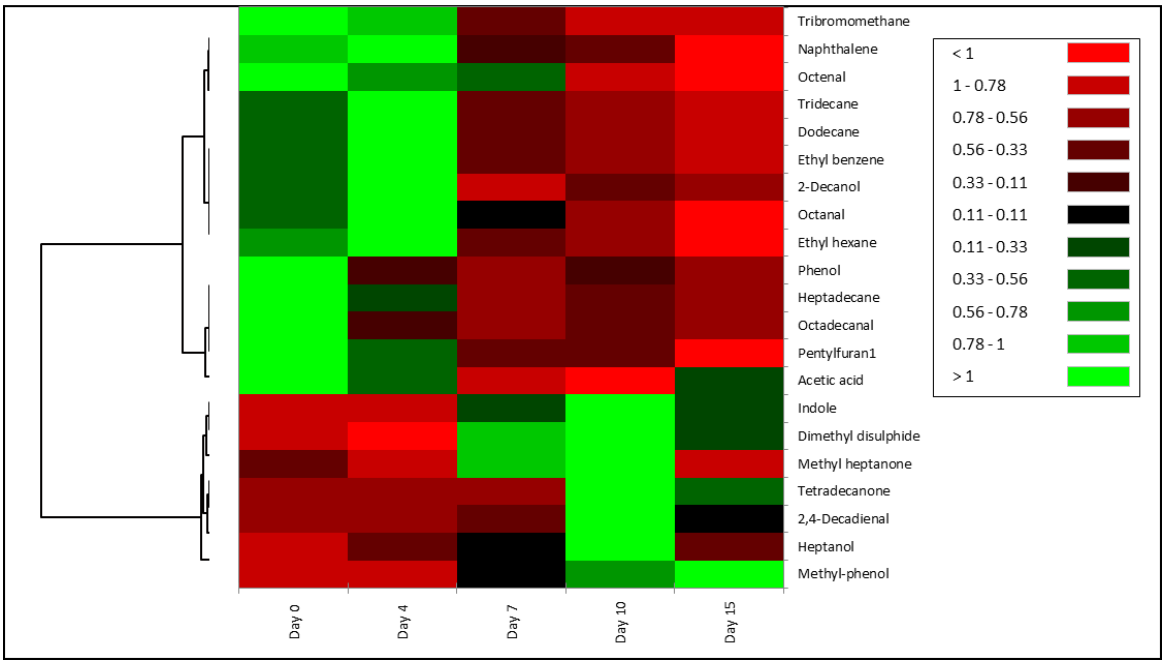


**Figure 3.19**: Heat-map of the evolution of identified volatile metabolites in commercial packs (pouch water) stored at 4°C for 15 days. Colour intensity indicates normalised amounts of volatiles with high concentration in red and low concentration in green.

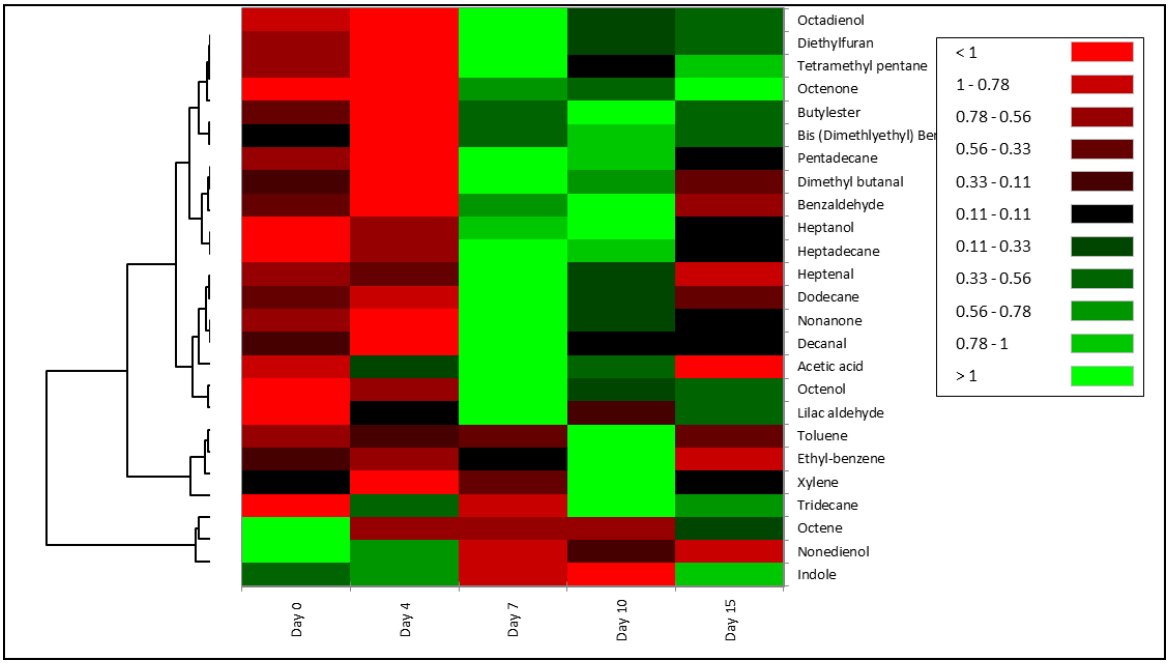


**Figure 3.20**: Heat-map of the evolution of identified volatile metabolites in commercial packs (mussel meat) stored at 4°C for 15 days. Colour intensity indicates normalised amounts of volatiles with high concentration in red and low concentration in green.

**Table 3.2:** Freshness and spoilage volatile metabolites in pouch water based on multivariate exploratory analysis.

| **Depurated** | | **Undepurated** | | **Commercial packs** | |
|---|---|---|---|---|---|
| Freshness | Spoilage | Freshness | Spoilage | Freshness | Spoilage |
| 3-Methylbutanol | 2-Ethylfuran | Lilac aldehyde | Methyl phenol | 2-Decanol | Methyl phenol |
| Octadienol | Fatty acid | Hexanal | Indole | Dodecane | Indole |
| 3,5 - Octadiene | Bis (Dimethylethyl) Benzene | Tribromomethane | Dimethyl disulphide | Ethyl hexane | Dimethyl disulphide |
| Tribromomethane | Dimethyl disulphide | Nonanal | Tetradecanone | Octanal | Tetradecanone |
| Ethyl benzene | Heptadecane | Methyl heptanone | Methyl heptanone | Octenal | Methyl heptanone |
| Hexanal | Octadiene | Nonanone | Heptanol | Tridecane | Heptanol |
| Nonanone | Pentylfuran2 | Tetramethyl pentane | Heptadecane | | Heptadecane |
| Nonedienol | Xylene | | | | |
| Octadecanal | Methyl heptanone | | | | |
| Tetramethyl pentane | | | | | |

**Table 3.3:** Freshness and spoilage volatile metabolites in mussel meat based on multivariate exploratory analysis.

| **Depurated** | | **Undepurated** | | **Commercial packs** | |
|---|---|---|---|---|---|
| **Freshness** | Spoilage | Freshness | Spoilage | Freshness | Spoilage |
| Dodecane | Tetramethyl pentane | Dodecane | Heptanol | Octene | Diethylfuran |
| Heptenal | Acetic acid | Tribromomethane | 2-Ethyl-5-methylfuran | Nonedienol | Octadienol |
| Hexanal | 3-Methylbutanol | Dimethyl trisulphide | Octadienol | Benzaldehyde | Octenone |
| Nonanal | Indole | Nonedienol | 3,5 - Octadiene | Ethyl hexane | Octenol |
| Octadecanal | Methyl phenol | Benzaldehyde | Indole | Pentadecane | Indole |
| Pentylfuran1 | Octadienol | Ethyl hexane | Dimethyl sulphide | | Bis (Dimethlyethyl) Benzene |
| Nonedienol | Octene | Pentadecane | Octadiene | | |
| Dodecane | Octenol | | | | |
| Tribromomethane | | | | | |
| Nonedienol | | | | | |
| Benzaldehyde | | | | | |
| Ethyl hexane | | | | | |
| Pentadecane | | | | | |
| Octadienol | | | | | |
| Octene | | | | | |

## 3.5 Discussion

This present study aimed to evaluate the microbial quality, freshness and spoilage volatile headspace metabolites of depurated and undepurated modified atmosphere packaged live for 15 days storage at 4°C using HS-SPME GC/MS to understand the evolution of volatile metabolites responsible for freshness and spoilage of live mussels. All the samples used in this study were packed with high initial headspace oxygen (80%).

### 3.5.1 Headspace analysis of MAP live mussels

One of the ways to enhance oxygen availability to live mussels was the use of MAP. The little decline in oxygen that was observed in depurated and undepurated mussel packs on day 4 could be due to diffusion and dissolution of oxygen in the hermetic plastic, pouch water and slow metabolic activity of the mussels as result of the low temperature. The decline in oxygen was more evident from day 7 to day 15 and was more pronounced in commercial packs compared to other treatments. It should be noted that between 41 – 48 mussels were used in the commercial packs compared to laboratory samples that were packed with only 6 – 10 mussels. Similarly, mussels in commercial packs (1 kg per pack) were depurated for 4 hr while laboratory packs mussels (200 g per pack) were depurated for 8 hr. In addition to this, mussels in the commercial packs were of more variable size found while mussels in the laboratory packed samples were within 61 - 80 mm in sizes. The result of commercial packs obtained on day 4 was similar to that of Pastoriza et al. (2004) who evaluated effect of high (75% and 85%) and low (20%) initial headspace oxygen on shelf-life and mortality of live

mussels. In their study, initial headspace oxygen (75% and 85%) reduced by 16% on day 4. The results of this study showed that mussels' mass, pack size and or duration of depuration contribute to the decline in oxygen.

The observed increase in $CO_2$ with storage days was expected due to mussels and microbes' respiration thereby creating decreasing available oxygen in the packs. Study on the rate of respiration in MAP live mussels is yet to be carried, therefore, it was difficult to correlate oxygen consumption with the increment of $CO_2$ during cold temperature storage. In MAP live clams, it was reported that the rate of respiration increases as $CO_2$ increases during cold storage (Ho et al., 1997). Pastoriza et al. (2004) observed 12.1% headspace $CO_2$ on day 4 in mussels packed with 75% headspace oxygen and stored for 6 days at 2 - 3°C. A similar result was observed in live clams with 70% initial headspace oxygen stored at > 5°C for 6 days (Goncalves et al., 2009). They observed 16.5% $O_2$ and 11% $CO_2$ on day 6.

### 3.5.2 Effects of mortality, physicochemical parameters and TVC on quality and shelf-life of MAP live mussels

Mortality of mussels as a quality parameter was observed in this study to correlate it with shelf-life and evolution of volatile metabolites. The first mortality was on day 7 in commercial packs compared to day 10 in other treatments could be a result of the decreased oxygen (42%) in the commercial packs when compared to other treatments. The high density of mussels in the commercial packs possibly resulted in decreased survival for oxygen and stress. Bernardez and Pastoriza (2011) reported high mortality in live mussels packed with

low oxygen (21%) and stored between 1 - 3°C. According to Pastoriza et al. (2004), stress resulting from the long processing period during packaging could lead to mortality of live mussels. In addition, the acid accumulation and increase in metabolite production are also factors considered to result in mortality of MAP live mussels (Bernardez and Pastoriza, 2011). Using less than 20% mortality baseline of Bernárdez and Pastoriza (2011), the marketable period of commercially packed mussels was day 7 while that of other treatments was 10 days. Pastoriza et al. (2004) reported that live mussels packed with high oxygen (75–80%) and stored for 6 days at 2 - 3 °C had 10% mortality. Increase in microbial load has also been reported to cause mortality in mussels (Eggermont et al., 2014).

In this current study, sterile fresh water was added to mimic industrial practice because it could facilitate survival in packaging and provides a humid environment for the packed mussels. However, due to the metabolic activities of the live mussels, by-products such as ammonia and faeces are released into the packs thereby providing a suitable medium for microbial growth which in turn influences pH. There was no significant difference ($p>0.05$).in the pH of mussel meat in all the treatments as stable pH (5.05 – 5.77) was observed from day 0 up until day 10. This was in accordance with the results obtained in both phase 2 and 3 of Chapter 2. This was similar to the observation of Bernardez and Pastoriza (2011) who reported the pH values of 6.6 – 6.7 in mussel meat in conjunction with the initial high $O_2$ concentration in packed live mussels. As stated by Pottinger (1948), pH can be used to classify the quality of bivalve shellfish meat to fresh (5.9 - 6.2), good (5.5 - 5.7) and spoilt (≤ 5.2). Using this scale, it shows that mussels used in this current study were still good at day 10 (pH: 5.3 - 5.5) in all the treatments. However, this needs further investigation because

shucked oysters were used in the study compared to this present study that used MAP live mussels. It was expected that the pH of mussel meat would decrease on day 15 because the mussels were dead; however, an increase in pH was noticed, possibly due to microbial metabolism. This contradicts the work of Aaraas et al. (2004) who observed the pH of dead oysters to be 5.2 - 5.4. According to Masniyom et al. (2011) a decrease in mussel meat pH could be a result of production of carbonic acid from respiration with mussel meat providing a buffering effect. Production of lactic acid from glycogen during storage due to decrease in headspace oxygen is another contributing factor to decrease in pH (Pacheco-Aguilar et al., 2008).

Freshly harvested and air-exposed mussels have been reported to accumulate ammonia within closed shell (Barrento et al., 2014). This could be released into the pouch water. The total ammonia nitrogen (TAN mg $L^{-1}$) was therefore monitored during this current study because accumulated ammonia within the mussels and released into the pouch water or headspace could become toxic to the mussels during storage. No ammonia was observed on day 0 in the pouch water. This could be because of slow metabolic rate arising from acclimatization to a new environment. As live mussels degrade, proteins are released which could be metabolised by spoilage bacteria to produce volatile metabolites. Protein catabolism in the mussels releases nitrogen that serves as a precursor to ammonia in seafood (Hawkins et al., 1992). As reported by Bernardez and Pastoriza (2011), such nitrogen would then be transported, excreted and diffused to the gills as a result of increased concentration, and this will become accumulated in the pouch water.

At the start of the experiment, the mean total viable count (TVC) of mussel meat in depurated samples was 3.32 ± 0.26 log CFU $g^{-1}$ similar to the result that was obtained in Chapter 2. However, this was lower than the results of Bernardez and Pastoriza (2011) who reported 4.18 log CFU $g^{-1}$ on day 0. The mean TVC in all treatment was lower than the microbial limit of 6 log CFU $g^{-1}$ for raw molluscan bivalve shellfish as recommended by national advisory committee (NAC) on microbiological criteria for foods (National Advisory Committee on Microbiological Criteria for Foods, 1992). The result obtained on day 4 in this current study, was similar to that obtained by Pastoriza et al. (2004) on day 6 of live mussels packed with 75% initial headspace oxygen and stored at 2 – 3°C. Aru et al. (2016b) reported similar results on day 6 of raw mussels stored at 4°C. In this current study, the mean TVC significantly increased on day 7 above microbial limit in mussel meat and pouch water samples of undepurated and commercial packs. Light objectionable spoilage (sulphide) smell was noticed on day 7 when both undepurated and commercial samples were opened. The smell became prominent on day 10 which was not observed in depurated packs. The observed smell could be attributed to microbial activities as this correlated with data on mortality showing release of sulphide and nitrogenous compounds. A microbial load of 7 – 8 Log CFU $g^{-1}$ has been correlated with production of objectionable smell by spoilage bacteria in seafood (Broekaert et al., 2013; Dalgaard, 1995a). Bernardez and Pastoriza (2013) attributed increased TVC in live mussels to organic waste, decomposed mussels and higher storage temperature (7°C). Dead mussels and pouch water provide a biological medium for spoilage bacteria and other microbes present in the mussels. Higher TVC in commercial packs and undepurated samples could also be attributed to the voiding of gut by mussels in the packs. The rejection time in this study for undepurated and commercial packs was 7 days

due to the objectionable smell which could be smelt from the pouch water or mussels. The TVC reported in depurated samples on day 15 in this study was similar to that obtained on day 15 in 80% $CO_2$ packed shucked mussels (6.8 ± 0.3 Log CFU $g^{-1}$) reported by Goulas et al. (2005).

Meat condition index (MCI) was evaluated in this study. MCI, also known as meat yield is usually used to evaluate the market quality and health of bivalve shellfish such as mussels, clams and oyster (Orban et al., 2002). It is affected by food availability, temperature, location and reproductive stage of the shellfish (Gallardi et al., 2014; Oliveira et al., 2015; Orban et al., 2002). Mussels that are not fed could utilise accumulated glycogen as a source of energy for respiration and spawning thereby reducing the market quality. No spawning was observed throughout this current study. The MCI in this study was stable throughout the storage period. No spawning was observed. Various studies on MCI of freshly harvested mussels have been reported. Orban et al. (2002) reported that seasonal variation affected the quality of mussels harvested from two separated sources in Italy. Gallardi et al. (2014) studied the effect of holding period on MCI of live blue mussels cultured in Canada and observed loss of weight in mussels held for longer than commercial practice because stored glycogen was used during this period.

### 3.5.3 Evolution of freshness and spoilage volatile metabolites of MAP live mussels during storage at 4°C

Volatile metabolites have been used over the years as indicators of freshness and spoilage of seafood due to the direct relationship of microbial, autolytic and chemical activities (Broekaert et al., 2013; Fuentes-Amaya et al., 2016; Mace et al., 2013b). However, there is dearth of literature on headspace volatile metabolites present in MAP live mussels. It was, therefore, important to investigate volatile metabolites in the headspace of both mussel meat and pouch water as indicators of either freshness or spoilage. As stated by Leduc et al. (2012) volatiles indicative of freshness and good quality decrease with storage while spoilage indicators increase. The majority of the volatile metabolites observed in this study have also been observed in other seafood such as oysters (Zhang et al., 2009), green shell mussels (Tuckey et al., 2013), and seabass (Vidal et al., 2017). Factors such as storage temperature and packaging conditions of either air, MAP or vacuum packaging, type of seafood (raw or processed) and microbial community determine the volatile metabolites present in seafood.

In this current study, freshness volatiles markers (mostly aldehydes, alcohols and ketones) identified included 2-decanol, benzaldehyde, dodecane, ethyl hexane, ethyl benzene, heptenal, hexanal, lilac aldehyde, nonanal, nonanone, nonedienol, octadecanal, octanal, octenal, pentadecane, tribromomethane and tridecane. Fuentes et al. (2009) also found 16 aldehydes in raw blue mussels stored at 4°C. The presence of aldehydes has been reported in other seafood. For example, aldehydes were reported in refrigerated (4°C) seabass stored for 15 days (Vidal et al., 2017). Aldehydes were present in all the treatments and commercial

packs in this current study. Studies indicated that aldehydes are products of either microbial activity or enzymatic carboxylation of unsaturated fatty acids in seafood and have been used to characterise seafood as fresh (Leduc et al., 2012; Selli and Cayhan, 2009). However, Parlapani et al. (2015) stated that aldehydes could also be because of microbial activities based on the results of their study in which they observed aldehydes produced by *Pseudomonas* spp., in fish samples. Aldehydes decreased with storage of mussels (Yasuhara, 1987). This was like the result obtained in this study. Some aldehydes detected on day 0 in both the treatments and the commercial packs decreased across the storage days suggesting that they are indicators of the fresh smell observed in the live blue mussels. For example, heptenal, lilac aldehyde and benzaldehyde were observed to decrease with storage days in both mussel meat and pouch water of the treatments and commercial packs. Some aldehydes such as hexanal increased with TVC during storage of the samples. This implies that while some aldehydes were products of enzymatic catabolism of unsaturated fatty acids in mussels, some resulted from microbial spoilage activities. Aldehydes were not only detected in mussel meat but were also found in the pouch water. The only means of aldehydes getting into the sterile freshwater that was added as pouch water was from the mussels. Hence, aldehydes could be said to be water-soluble or released with intervalvular fluid during the packaging process and could affect either freshness or spoilage of mussels.

In this current study, the presence of alcohol was also observed in the samples. Fuentes et al. (2009) reported presence of alcohol in raw mussels. As stated by Fuentes et al. (2009), only unsaturated alcohols contribute to the flavour of mussels compared to saturated alcohols that do not contribute to mussel flavour.

Previous studies have reported the presence of ketones in fresh mussels (Yasuhara, 1987; Yasuhara and Morita, 1987). Ketones have been reported in other shellfish such as oysters (van Houcke et al., 2016; Zhang et al., 2009) and clams (Zhang et al., 2010). In this study, nonanone was present in the mussel meat samples of treatments and commercial packs until the end of commercial shelf-life. Hence, nonanone may have contributed mussel flavour.

Spoilage volatile metabolites identified included 2-ethylfuran, bis (dimethylethyl) benzene, butyl ester, diethyl furan, dimethyl sulphide, dimethyl disulphide, heptadecane, heptanol, indole, methyl phenol, octadiene, octenol, octenone, tetradecanone, and xylene. Indole as nitrogen volatile was detected in this study. It has been attributed to enzymatic catabolism and or to microbial breakdown of seafood protein and nucleotides (Limbo et al., 2009). Additionally, excretion and release of TAN into the packs could serve as precursor for production of nitrogen compounds such as indole which increased with the storage days. Since TAN excreted into the pouch water could serve as a suitable medium for microbial growth, this could account for the increased presence of dimethylamine in the pouch water. Similarly, accumulated TAN could become toxic to the mussels thereby contributing to stress and mortality during storage.

When seafood deteriorates, sulphur compounds are the major volatiles released because seafood contains a high amount of amino acids such as cysteine and methionine that are precursors to sulphide volatile metabolites such as dimethyl sulphide (Grigorakis et al., 2003;

Varlet and Fernandez, 2010). In addition, seafood spoilage bacteria such as *Pseudoalteromonas* spp. can produce sulphur compounds such as dimethyl sulphide in seafood (Broekaert et al., 2013). These volatile metabolites usually manifest as first indicators of spoilage seafood (Broekaert et al., 2013). Dimethyl sulphide and dimethyl disulphide were detected in this study. The presence of sulphur compounds such as dimethyl sulphide in mussels is due to microbial degradation of mussel meat and (Tuckey et al., 2013). For example, an increase in dimethyl sulphide across the storage days was observed in all the treatments and commercial packs. Dimethyl sulphide was detected on from day 4 while dimethyl disulphide appeared on from day 10 in depurated mussels and day 7 in both undepurated and commercial packs. This correlated with shelf-life and the onset of an objectionable smell. Early mortality observed in commercial packs may have contributed to the presence of all the four sulphur compounds. This resulted in an early rejection of the samples on day 7. Similarly, mortality contributed to the increased TVC (> 7 log CFU $g^{-1}$) from day 7 in the commercial packs. Degradation of dead mussels provides a suitable medium for microbial growth, leading to increased TVC and increased volatile metabolites production. This was in support of the observation of Broekaert et al. (2013) that 7 – 8 log CFU $g^{-1}$ of TVC is required for production spoilage volatile metabolites in shrimps. Sulphur compounds have also been reported in cooked mussel meat (Guen et al., 2000). This buttresses the fact that production of sulphur compounds in mussels could be either by microbial and/or by autolytic activities.

In this study, butyl ester was the major ester detected in the samples and was mostly present in mussel meat. As TVC increases, butyl ester also increased in the samples. According to

Parlapani et al. (2017), the formation of esters in seafood is caused by metabolic activities of spoilage bacteria such as *Pseudomonas* spp., and lactic acid bacteria (LAB). In a recent study, Parlapani et al. (2015) reported the production of esters in MAP gutted - sea bass due to the metabolic activities of pseudomonads which increased with TVC. However, due to the focus of this study, no attempt was made to isolate any of the bacteria.

The presence of aromatic hydrocarbons in mussels has been attributed to contamination of culture water with petroleum (Fuentes et al., 2009). In this current study, mussels were harvested from a port water with few anthropogenic activities. However, detection of aromatic compounds in this study could be attributed to the trays that were used for packing the mussels as the tray are made of plastic. As observed in the depurated mussels, aromatic volatiles were more abundant in mussel meat across the storage days compared to pouch water with more volatiles towards the end of commercial shelf – life and on day 15. Aromatic volatile metabolites were more abundant in the mussel meat than pouch water as expected.

Although more hydrocarbons were detected in the mussel meat, they were more persistent in the pouch water. Hydrocarbons are not odour active in cooked mussel meat (Guen et al., 2000). Hence, there is need to use Gas chromatography–olfactometry to ascertain the impact of hydrocarbons on mussel freshness. In this current study, the source of hydrocarbons in the mussel meat could be also attributed to trays used for packing the mussels.

## 3.6 Conclusion

This study evaluated the microbial quality, freshness and spoilage volatile headspace metabolites of depurated and undepurated modified atmosphere packaged live mussels stored at 4°C for 15 days using HS-SPME GC/MS. The study showed that HS-SPME GC/MS is a useful method for evaluating freshness and spoilage volatile metabolites of live mussels during storage. The identified markers for freshness or spoilage and provided information on the effect of depuration on freshness or spoilage of live mussels. This could help in the development of quality improving and monitoring technologies that could extend the shelf-life of MAP live mussels. Similarly, multivariate exploratory analysis (PCA and heat map) can be used to analyse complex data generated with HS-SPME GC/MS. The decline in headspace oxygen and metabolic by-products of mussels are contributing factors to mortality of MAP live mussels. Freshness volatiles such as hexanal, heptanal, and octanal decreased with storage. Spoilage volatiles such as dimethyl trisulphide, decanal, phenol and octadienol increased with storage. The shelf-life of MAP live mussels in the studies reported in literature ranged between 6 and 8 days. However, in this current study, the shelf-life of live mussels packed with 80% oxygen was observed to be 10 days. In this current study, the storage temperature was 4°C throughout the experiment while previously reported storage temperature was between 1 – 7°C and the headspace was also between 75 – 83% oxygen. Hence, initial headspace oxygen and storage temperature play a significant role in the shelf-life of live mussels. The freshness and spoilage volatiles reported in this current study were also reported in the spoilage of mussels and other seafood (Parlapani et al., 2014; Parlapani et al., 2015; Yasuhara, 1987; Zhang et al., 2010; Zhang et al., 2009). It was observed that more than one volatile metabolite could be used in the assessment of freshness and spoilage

of MAP live mussels. However, further studies to the understand effect of depuration on spoilage microbial community and succession is suggested. There is need to study the contribution of early mortality on objectionable smell in addition to comparative studies of the correlation between autolytic and microbiological spoilage mechanisms. Lastly, study on the contribution of hermetic container towards production of aromatic compounds in packaged mussels is suggested.

# Chapter 4: Spoilage microbial community profiling by 16S rRNA amplicon sequencing of modified atmosphere packaged live mussels stored at 4°C

## 4.1 Introduction

There is an increasing demand for live mussels due to freshness and palatability (Pastoriza et al., 2004). Modified atmosphere packaging (MAP) has been used for preservation of live mussel till consumption (Pastoriza et al., 2004).

Farming environment and post-harvest handling and storage impact the microbiota of shellfish (Lee et al., 2016). Depuration is a post-harvest handling process applicable to shellfish (Barile et al., 2009) to purge their gut contents and associated microorganisms in static seawater over a period of time prior to MAP packing (Ramos et al., 2012). However, during storage, changes in the metabolic state of the shellfish, microbial community and/or physiochemical conditions within the MAP product could lead to spoilage (Lee et al., 2016).

Culture-dependent methods such as agar culturing and biochemical characterisation have been used for microbial analysis of seafood including mussels (Caglak et al., 2008; Goulas and Kontominas, 2005; Pastoriza et al., 2004). However, these methods cannot give a complete understanding of microbial community succession during storage due to the limitations of culturing, that underestimate microbial diversity and/or provide an incomplete view of the microbial community contributing to product spoilage (Parlapani and Boziaris, 2016; Powell and Tamplin, 2012).

To overcome these limitations, culture-independent methods are often used. For example, Fernandez-Piquer et al. (2012) used terminal restriction fragment length polymorphism (T-

RFLP) targeting 16S rRNA gene to investigate the impact of post-harvest storage temperature (4 - 30°C) on the microbial community in live Pacific oysters (*Crassostrea gigas*). They reported high microbial diversity and that *Psychrilyobacter*, a genus from the phylum Fusobacteria was more dominant at 4°C while Bacteroidetes were dominant at 30°C indicating the effect of storage temperature on microbial diversity. Microbial diversity profiling using V1 - V3 regions of 16S rRNA gene has been used to study the spoilage microbiota of half-shell oysters stored at 4°C (Madigan et al., 2014). They observed that *Pseudoalteromonas* and *Vibrio* from the phylum Proteobacteria were dominant genera at the end of storage.

Several studies have concluded that packaging and storage conditions play significant roles in the microbial diversity of shellfish (Fernandez-Piquer et al., 2012; Madigan et al., 2014). However, there is little information on the microbial community-associated with MAP live mussels or how pre-packaging depuration modifies the microbial community and spoilage. This study therefore aimed to use 16S rRNA amplicon sequencing (Next Generation Sequencing – NGS) to investigate microbial community succession of MAP depurated and undepurated live mussels packed with high initial oxygen (80% $O_2$ / 20% $N_2$) and stored at 4°C for 15 days.

## 4.2 Materials and methods

### 4.2.1 Sample collection

Freshly harvested mussels were collected from commercial mussel farm in the East Coast of Tasmania, Australia as stated in Chapters 2 and 3. Mussels were washed, cleaned and debearded before grading. Mussels graded as commercial size were then transported into the laboratory in iced-packed Styrofoam boxes as described in Chapter 3.

### 4.2.2 Mussel packaging and storage

The mussels were randomly divided into two sub-samples. One sub-sample was subjected to 8 hrs depuration under the optimised depuration conditions described in Chapter 2. Briefly, 6 – 8 mussels were packed with 80% $O_2$ and 20% $N_2$ as described in Chapter 3. The second sub-sample was not depurated but stored for 8 hrs at 4°C and then packed in the same manner as the depurated mussels. Thereafter, samples were stored at 4°C for 15 days. The commercially-depurated mussels used as reference were depurated for 2 hr and MAP-packed with each pack containing 35 – 42 mussels (1kg) and stored at 4°C for 15 days as described in Chapters 2 and 3.

### 4.2.3 Microbial diversity profiling

The MAP-packaged mussels and pouch water were sampled on day 0, 4, 7, 10 and 15. To prevent any anomaly in the microbial diversity that would be observed in the samples, random triplicate packs of both treatments and control were opened and sacrificed at each

time point before preparing the samples for NGS-based microbial diversity profiling based on 16S rDNA sequencing. Twenty grams of mussel meat was homogenised with 180 mL sterile alkaline peptone water for 60 seconds in a sterile tube (Dabade et al., 2015). The pouch water was mixed prior to sub sampling before 40 mL from the packs was collected in a sterile falcon tube (50 mL). Duplicate meat and pouch water samples were prepared and immediately frozen and stored at - 80°C until further analysis.

The bacterial genomic DNA from both mussel meat and pouch water was extracted using a bead milling step with the PowerLyzer Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) as described by Nielsen et al. (2018). Polymerase chain reaction (PCR) - based Quality control (QC) was performed on the extracted DNA samples to ascertain PCR inhibition of samples and samples showing insufficient amplification were re-extracted to obtain a minimum of 30 000 reads. The DNA extraction, PCR amplification and sequencing were performed by the Australian Genome Research Facility in Brisbane.

The V1- V3 hypervariable and flanking regions of 16S rRNA gene were amplified using primers 27F (AGAGTTTGATCMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG) (Madigan et al., 2014). AmpliTaq Gold 360 master mix (Life Technologies, Australia) was used for the primary PCR amplification based on 95°C for 7 minutes followed by 29 cycles of: 94°C for 45 seconds, annealing at 50°C for 60 seconds extension at 70°C for 60 seconds; followed by 72°C for 7 minutes. A secondary amplicon indexing using PCR (TaKaRa Taq DNA Polymerase, Clontech) was used while the amplicon concentration was then normalised by fluorometry (Invitrogen, Picogreen) and the samples were combined into equimolar pools prior to

sequencing on an Illumina MiSeq instrument (San Diego, CA, USA) with 2 x 300 bp paired-end chemistry. Image analysis was performed in real-time by the MiSeq Control Software (MCS) v 2.6.2.1 and Real Time Analysis (RTA) v1.18.54 performs real-time base calling on the MiSeq instrument computer. Then the Illumina bcl2fastq 2.19.1.403 pipeline was used to generate the sequence data. Paired-end reads were assembled by aligning the forward and reverse reads using PEAR, version 0.9.5 (Zhang et al., 2014).

### 4.2.4 Bioinformatics

Priming sequences were identified, trimmed and processed using Quantitative Insights into Microbial Ecology - QIIME 1.8 (Caporaso et al., 2010), USEARCH, version 8.0.1623 (Chen et al., 2011; Edgar, 2010) and UPARSE software (Edgar, 2013). Using search tools, sequences were quality filtered, full-length duplicate sequences were removed and sorted by abundance. Singletons or unique reads were discarded. Sequences were clustered followed by chimera filtering using "RDP_gold" database as a reference. To obtain number of reads in each operational taxonomic unit (OTU), reads were mapped back to OTUs with a minimum identity of 97% (Liu and Tong, 2017). Taxonomy was assigned using the Green genes database, version 13_8 (Chen et al., 2011). The data obtained was pooled from the duplicate samples for each sampling day. Venn diagrams were used to show shared and unique OTUs (Li et al., 2016). Heat-maps were constructed to visualize microbial community changes during storage time for the mussel meat and pouch water samples for depurated, undepurated and commercial packed mussels (Zhang and Wang, 2017). The dominant bacteria were defined in the legend of the heat-map. Abundance > 50% was further termed dominant.

### 4.2.5 Diversity analyses

The microbial species richness, diversity indices (Chao 1 estimator and Shannon index) and rarefaction curves were analysed using QIIME (Caporaso et al., 2010; Song et al., 2016). The datasets for metagenomic sequencing were deposited in the Sequence Read Archive (SRA) database and are available under NCBI Submission ID: SRA accession: SRP 144357, Bio Sample accession: SAMN 09011654 and Bio Project ID: PRJNA 454672. (https://www.ncbi.nlm.nih.gov/bioproject/454691). Abundance > 50% was used as the criterion for the term "dominant" in the Chapter.

### 4.2.6 Statistical analysis

To identify the main taxa that are changing in the samples, two-way analysis of variance (ANOVA) based on Tukey's multiple comparison tests (data was not normally distributed) was carried out at 95% confidence interval (Xia and Sun, 2017). The analysis was carried out using GraphPad Prism 7.05 version (GraphPad Software, CA 92037 USA).

## 4.3 Results

### 4.3.1 Operational taxonomic units (OTUs) and Taxonomic species richness in mussel meat

The rarefaction curves of mussel meat from the undepurated mussels at the sampling points plateaued indicating sufficient sampling reads (Figure 4.1). Day 0 had highest microbial diversity. Days 7 and 10 showed similar microbial diversity but lower diversity was observed on day 15.

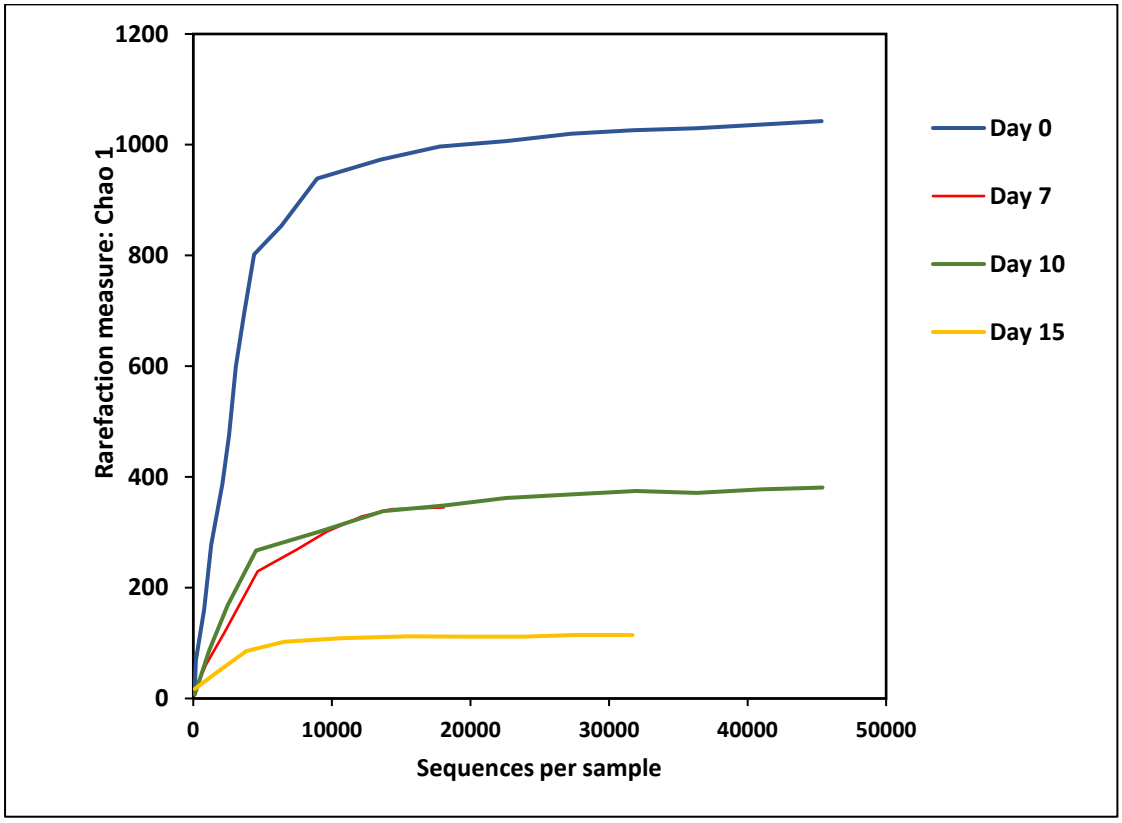


**Figure 4.1:** Rarefaction curve indicating the number of OTUs in mussel meat of undepurated mussels based on V1-V3 of 16S rRNA gene sequencing at > 97% sequence similarity. Different colours indicate sampling days. The Chao1 estimator (y- axis) indicates the number of OTUs (taxa) while (x-axis) represents the population of bacteria in the sample.

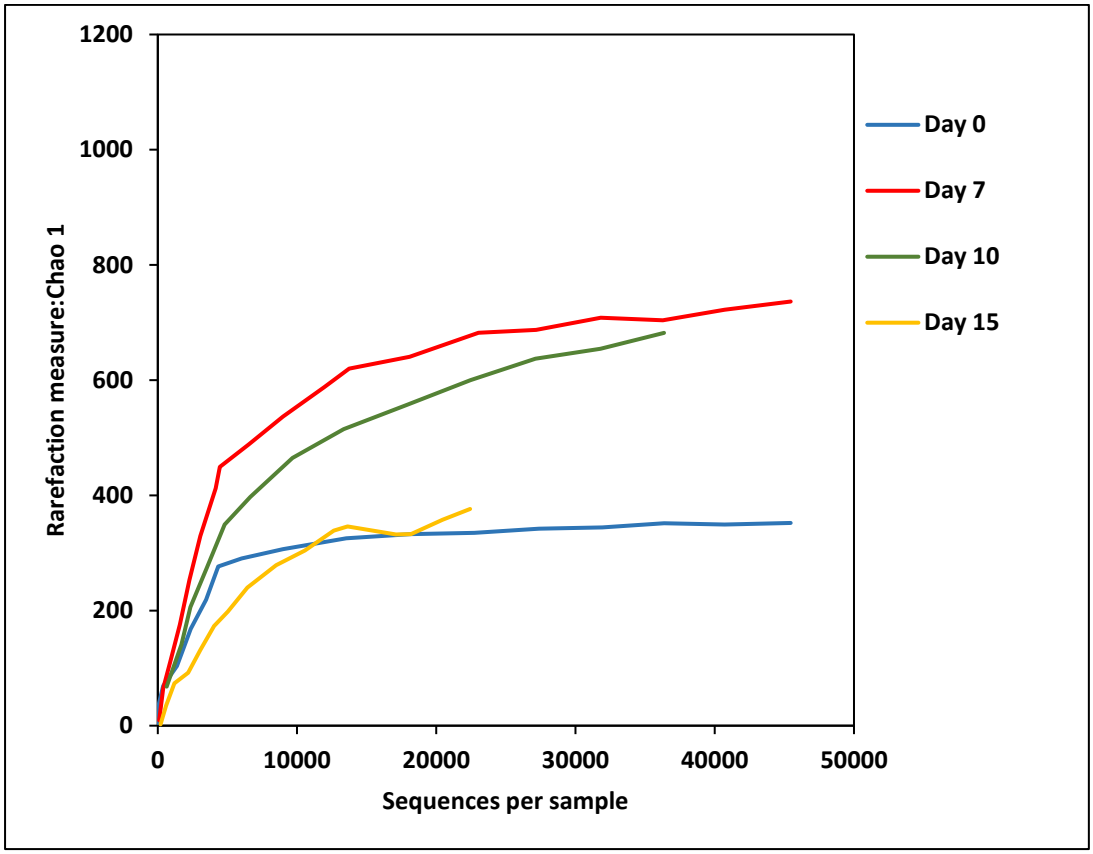


**Figure 4.2**: Rarefaction curve indicating the number of OTUs in mussel meat of depurated mussels based on V1-V3 of 16S rRNA gene sequencing at > 97% sequence similarity. Different colours indicate sampling days. The Chao1 estimator (y- axis) indicates the number of OTUs (taxa) while (x-axis) represents the population of bacteria in the sample.

The curves on days 0, 7 and 15 in the depurated mussels plateaued, indicating sufficient sampling reads. When compared with undepurated mussels, there were fewer OTUs and diversity in depurated mussels on day 0. The curve on day 10 was still approaching a plateau indicating insufficient sampling reads of the different bacteria in the original population (Figure 4.2). The microbial community observed on day 0 had significantly fewer OTUs than on days 7 and 10. However, although days 7 and 10 were diverse, day 7 was more diverse than day 10.

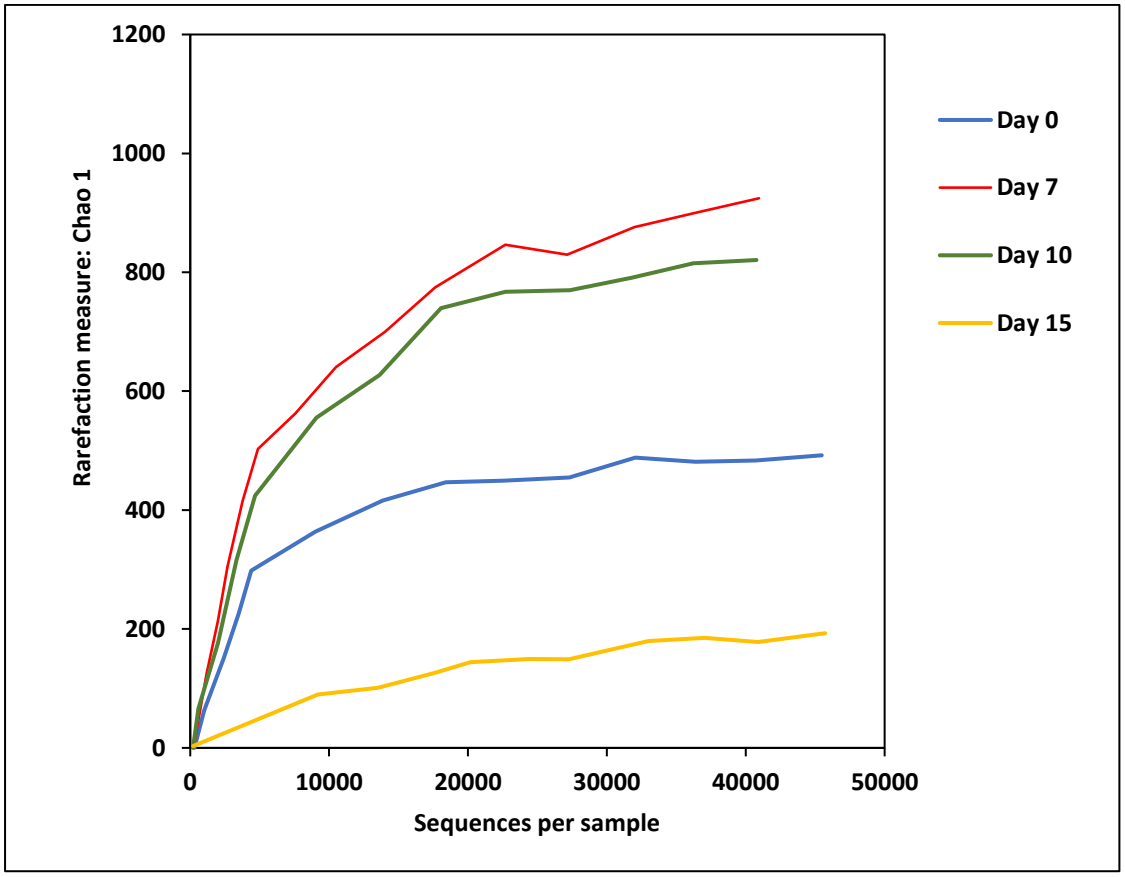


**Figure 4.3**: Rarefaction curve indicating the number of OTUs in mussel meat of commercially-depurated mussels based on V1-V3 of 16S rRNA gene sequencing at > 97% sequence similarity. Different colours indicate sampling days. The Chao1 estimator (y- axis) indicates the number of OTUs (taxa) while (x-axis) represents the population of bacteria in the sample.

In the commercially-depurated mussels, the curves showed a similar pattern to depurated mussels (Figure 4.3). However, they required more OTUs to level off than both undepurated and depurated mussels, indicating greater diversity. When the OTUs obtained in commercially-depurated mussels were compared with undepurated (Figure 4.1) and depurated mussels (Figure 4.2), it was observed that the undepurated mussels had the highest day 0 count, commercially-depurated mussels had highest on day 7 and 10 while depurated mussels had the highest on day 15. In commercially-depurated mussels, day 15 levelled off with fewer OTUs when compared with depurated mussels but higher than undepurated mussels. It should be noted that commercially-depurated (1kg packs

containing an average of 35 – 40 individual mussels compared with 6 – 8 mussels - 250 g of undepurated and depurated packs that were packed in the laboratory) mussels were depurated for only 2 hr.

**Table 4.1:** Operational taxonomic units (OTU) and species richness estimation (at minimum identity of 97% similarity) of mussel meat of undepurated, depurated and commercially-depurated mussels stored at 4°C for 15 days.

| Sample | Storage days | Sequences (n) | Average length (bp) | Reads (n) | Chao1 estimator | Shannon index |
|---|---|---|---|---|---|---|
| Undepurated | Day 0 | 220470 | 494 | 90291 | 1059 | 5.59 |
| | Day 7 | 94073 | 530 | 24422 | 346 | 2.34 |
| | Day 10 | 117356 | 518 | 47926 | 380 | 2.75 |
| | Day 15 | 117828 | 527 | 35424 | 110 | 2.78 |
| Depurated | Day 0 | 2353634 | 488 | 114090 | 373 | 3.78 |
| | Day 7 | 134084 | 505 | 55332 | 768 | 3.79 |
| | Day 10 | 101671 | 498 | 39474 | 718 | 3.74 |
| | Day 15 | 139290 | 536 | 24469 | 398 | 2.06 |
| Commercial | Day 0 | 102907 | 512 | 37040 | 392 | 2.71 |
| | Day 7 | 81062 | 514 | 23462 | 715 | 4.37 |
| | Day 10 | 90623 | 517 | 24501 | 639 | 4.62 |
| | Day 15 | 89328 | 526 | 37294 | 182 | 1.33 |

The total valid sequence reads obtained in the mussel meat of undepurated mussels was 549,727 (Table 4.1). The range of sequence reads was between 94,073 and 220,470 with the average length of the sequences being 518 base pairs (bp) (494 - 530) in undepurated. There was variation in the number of reads obtained across the sampling days. The taxonomy analysis based on Ribosomal Database Project (RDP) classifier yielded between 90,291 (day 0) and 24,422 (day 7) reads per sample across sampling days in undepurated mussels.

Taxon richness and diversity at day 0 was higher in the mussel meat of undepurated mussels than depurated or commercially-depurated mussels (Table 4.1). Both richness and diversity

reduced substantially at days 7 and 10 and was lowest on day 15 (110) (Table 4.1). Depurated and commercially-depurated mussel meat showed lower initial taxon richness and diversity, increased taxon richness at day 7, declining on day 10 and day 15. Diversity was lowest on day 15 in both depurated samples. The Shannon index indicated highest microbial diversity on day 0, least on day 7. However, there was not much difference in diversity between day 10 and 15.

### 4.3.2 Operational taxonomic units (OTUs) and Taxonomic species richness in pouch water

All the rarefaction curves of pouch water from undepurated mussels plateaued indicating sufficient sampling reads although day 15 had lowest sequences per sample (Figures 4.4). Day 0 had the most OTUs while day 7 showed fewest OTUs. An apparent increase in the OTUs was observed on day 10 but slightly decreased on day 15.

In the depurated mussels, the pattern of curves showed similar numbers of OTUs except that the curve did not plateau on day 0 compared to other days at 27261 sequences (Figure 4.5).

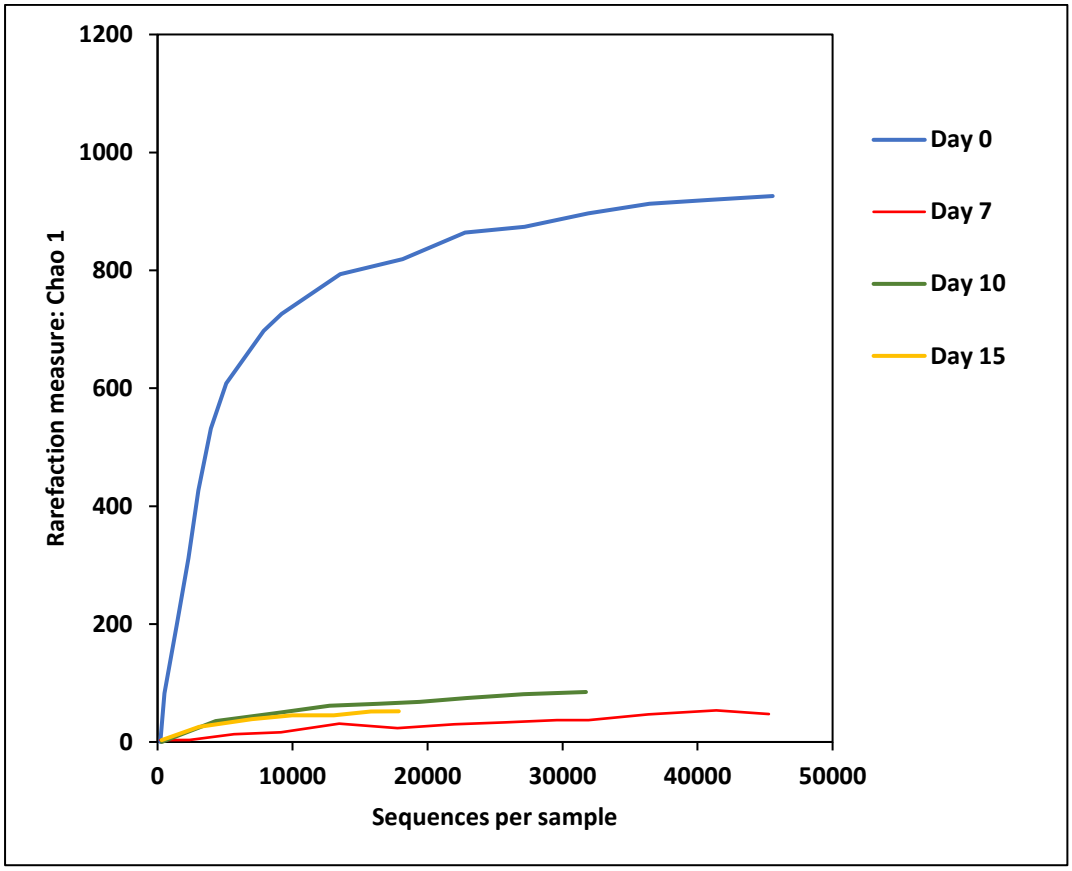


**Figure 4.4**: Rarefaction curve indicating the number of OTUs in pouch water of undepurated mussels based on V1-V3 of 16S rRNA gene sequencing at > 97% sequence similarity. Different colours indicate sampling days. The Chao1 estimator (y- axis) indicates the number of OTUs (taxa) while (x-axis) represents the population of bacteria in the sample.

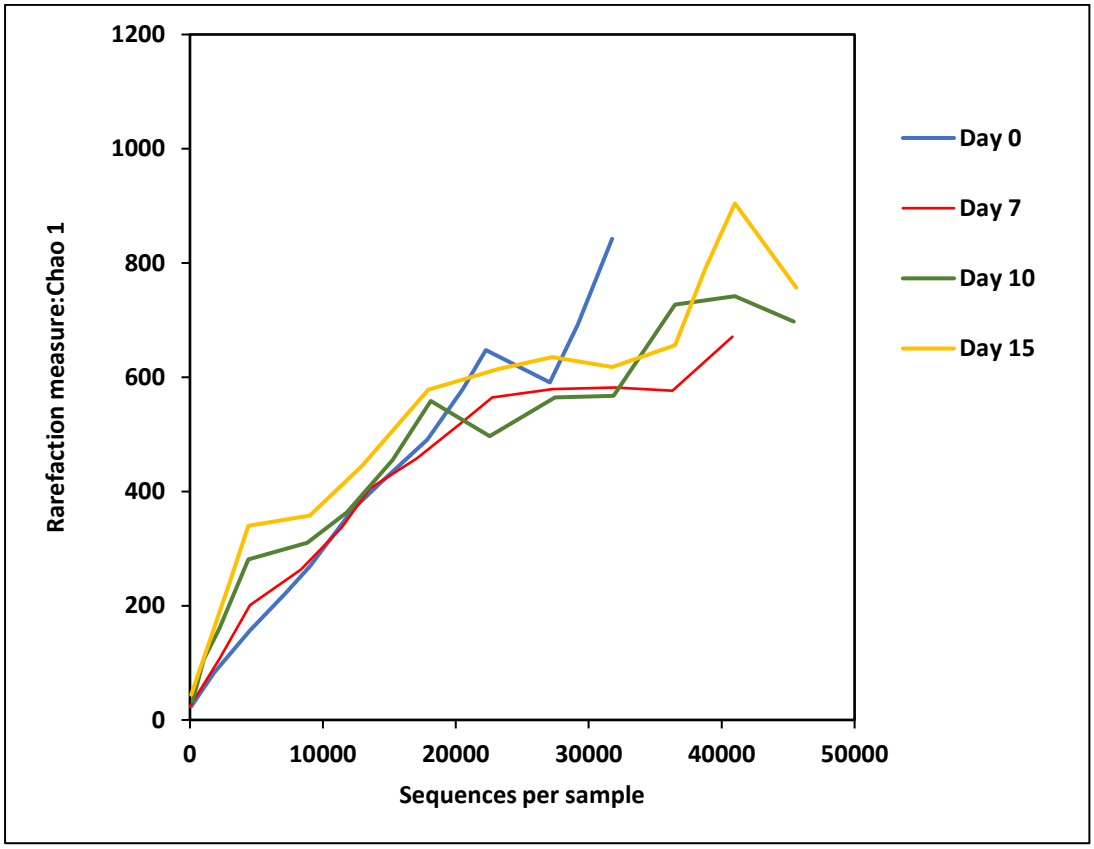


**Figure 4.5**: Rarefaction curve indicating the number of OTUs in pouch water of depurated mussels based on V1-V3 of 16S rRNA gene sequencing at > 97% sequence similarity. Different colours indicate sampling days. The Chao1 estimator (y- axis) indicates the number of OTUs (taxa) while (x-axis) represents the population of bacteria in the sample.

The curves in commercially packed mussels also showed similar pattern of microbial diversity to depurated mussels (Figure 4.6). However, the curve plateaued on day 0 and not on other days at 27461 sequences.

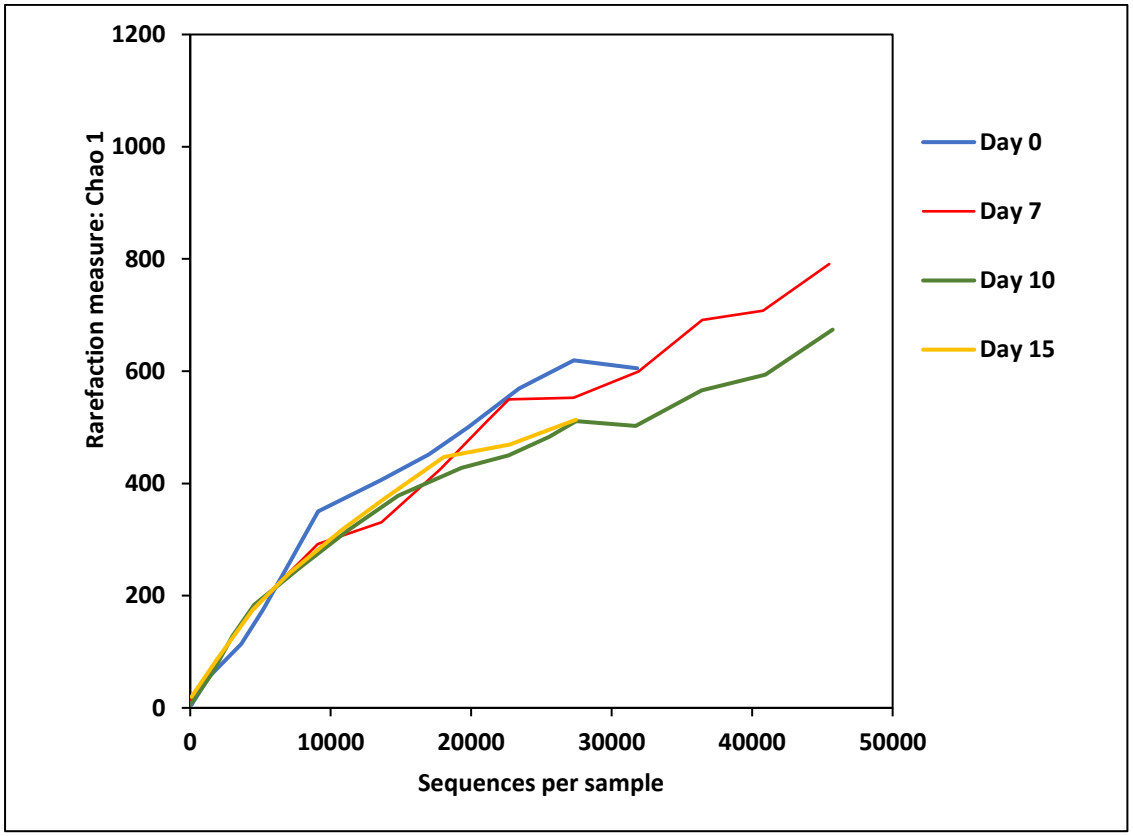


**Figure 4.6**: Rarefaction curve indicating the number of OTUs in pouch water of commercially-depurated mussels based on V1-V3 of 16S rRNA gene sequencing at > 97% sequence similarity. Different colours indicate sampling days. The Chao1 estimator (y- axis) indicates the number of OTUs (taxa) while (x-axis) represents the population of bacteria in the sample.

**Table 4.2:** Operational taxonomic units (OTU) and species richness estimation (at minimum identity of 97% similarity) of pouch water of undepurated mussels, depurated mussels and commercially-depurated mussels stored at 4°C for 15 days.

| Sample | Storage days | Sequence (n) | Average length (bp) | Reads (n) | Chao1 diversity | Shannon index |
|---|---|---|---|---|---|---|
| Undepurated | Day 0 | 119996 | 512 | 43690 | 1279 | 4.71 |
| | Day 7 | 83986 | 525 | 40953 | 70 | 1.09 |
| | Day 10 | 89643 | 524 | 31108 | 132 | 2.00 |
| | Day 15 | 57907 | 530 | 14236 | 79 | 2.42 |
| Depurated | Day 0 | 69410 | 524 | 25625 | 120 | 1.57 |
| | Day 7 | 71753 | 525 | 31428 | 93 | 1.28 |
| | Day 10 | 138138 | 522 | 52222 | 111 | 1.79 |
| | Day 15 | 100653 | 524 | 39776 | 118 | 1.76 |
| Commercial | Day 0 | 72105 | 524 | 25798 | 250 | 1.23 |
| | Day 7 | 86686 | 525 | 37308 | 335 | 1.26 |
| | Day 10 | 127402 | 523 | 45230 | 303 | 2.09 |
| | Day 15 | 94830 | 530 | 23176 | 208 | 2.85 |

In the pouch water of undepurated mussels, a total of 351,532 valid sequence reads ranging between 57,907 on day 15 and 119,996 on day 0 were obtained (Table 4.2). The average length of the sequences was 522.60 bp (511 – 529). RDP classifier - based taxonomy analysis yielded between 14,236 reads (lowest) on day 0 to 43,690 reads (highest) on day 15. Microbial community richness was least on day 7 and highest on day 0. Microbial community diversity was least on day 7 and highest on day 0 as shown by the Shannon index.

Taxon richness and diversity at day 0 was higher in the pouch water of undepurated than depurated or commercially-depurated mussels (Table 4.2) similar to what was observed in the mussel meat. Microbial richness and diversity reduced substantially on days 7 but slightly and apparently increase on 10 and was lowest on day 15 (79). The pouch water of both depurated and commercially-depurated mussel showed lower initial taxon richness and

diversity, decreased taxon richness on day 7 in depurated but apparently increased in commercially-depurated mussels, increasing till end of storage in depurated but declining on day 10 and day 15 in commercially-depurated mussels. Diversity apparently increased with storage in both treatments and was highest on day 15 in commercially-depurated mussels.

### 4.3.3 Bacterial community diversity and succession in mussel meat at phylum and genus levels

In the undepurated mussel meat samples on day 0, 22 phyla were observed but this decreased to 15 on day 7 and subsequently remained approximately the same number (Table 4.3). In the meat of commercially-depurated mussels, 25 phyla were observed on day 0, but this decreased to 18 on day 7 and further decreased to 13 on day 15 (Table 4.3).

**Table 4.3:** The distribution of OTUs at different levels in the mussel meat of undepurated, depurated and commercially-depurated mussels stored at 4°C for 15 days.

| Sample | Storage days | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|
| Undepurated | Day 0 | 22 | 55 | 105 | 177 | 243 |
| | Day 7 | 15 | 29 | 52 | 84 | 118 |
| | Day 10 | 16 | 34 | 64 | 102 | 136 |
| | Day 15 | 14 | 25 | 39 | 54 | 64 |
| Depurated | Day 0 | 16 | 37 | 71 | 113 | 144 |
| | Day 7 | 19 | 48 | 94 | 155 | 210 |
| | Day 10 | 17 | 40 | 76 | 127 | 172 |
| | Day 15 | 13 | 32 | 57 | 89 | 116 |
| Commercial | Day 0 | 25 | 33 | 62 | 95 | 124 |
| | Day 7 | 18 | 40 | 79 | 128 | 172 |
| | Day 10 | 17 | 39 | 74 | 116 | 158 |
| | Day 15 | 13 | 21 | 33 | 49 | 54 |

The number of genera was highest in undepurated mussel meat at day 0 compared to day 7 in depurated and commercially-depurated mussels. The number of genera decreased on days 10 and 15 (Table 4.3). More OTUs were observed on day 7 in both depurated and commercially-depurated mussels. The least OTUs were observed on day 15 in the treatments and commercially-depurated mussels. Phylogenetic composition of mussel meat samples varied considerably among sampling days.

Most phyla detected were unique to each sample day, and only 1 - 3 shared phyla between sampling days for undepurated mussel meat samples (Figure 4.7A). There were 20 unique OTUs on day 0, 10 on day 7, 13 on day 10 and 10 on day 15. There was no OTU shared across all sample days at the phylum level in the depurated mussels although 15 unique OTUs on day 0, 16 on day 7, 12 on day 10 and 11 on day 15 were observed (Figure 4.7B). However, undepurated mussels had more OTUs on day 0 than depurated mussels. In the

commercially-depurated mussels, few phyla were shared amongst some of the sample days, but mostly the phyla were unique to the sample day, with the total number of phyla declining with storage (Figure 4.7C). No OTU was shared across all the storage days. There were 22 unique OTUs on day 0, 12 on day 7 and 10 and 6 on day 15.

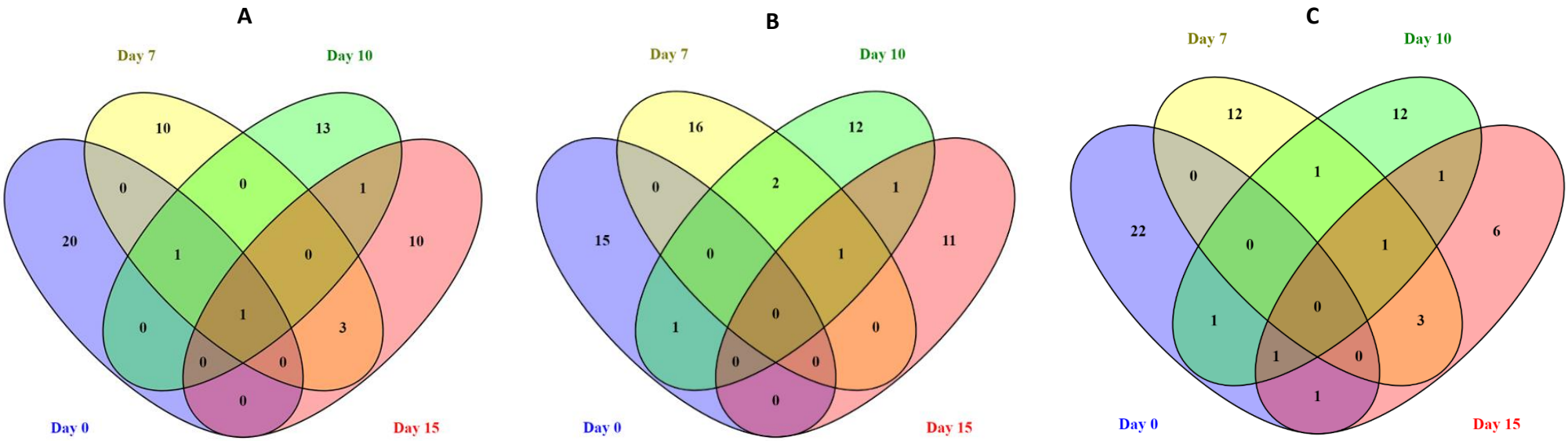


**Figure 4.7**: Venn diagram showing the distribution (97% similarity) of the OTUs at phylum level present in the mussel meat samples of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. Each number indicates shared or unique OTUs in the samples in each sampling day. Different colour indicates different sampling days (day 0 = blue, day 7 = yellow, day 10 = green and day 15 = pink).

The heat-map below shows the change in microbial community structure in the mussel meat at the phylum level during storage of live mussels as shown by the colour intensity of relative abundance (Figure 4.8). In the mussel meat of undepurated mussels, a total of 13 known phyla were observed (Figure 4.8A). Among these phyla, 5 (Proteobacteria, Cyanobacteria, Actinobacteria, Planctomycetes and Bacteroidetes) occurred throughout the storage period. On day 0, 10 phyla were observed among which Cyanobacteria (44%) and Proteobacteria (38%) were more dominant. The total number of dominant phyla decreased to 6 on day 7, however, no additional phylum was identified on day 7. Proteobacteria apparently increased on day 7 (93%) while Cyanobacteria decreased to 6%. Nine phyla were dominant on day 10 and 15 of which Proteobacteria remained most dominant on day 10 (84%) and on day 15 (92%). Some Cyanobacteria remained until day 15 (3%) and it was observed that both Fusobacteria and Spirochaetes appeared on day 10 and 15.

Figure 4.8B shows the relative abundance of identifiable phyla in depurated mussels. Unknown phyla represented only 0.27 – 1.35% of the community present in depurated mussel meat across the storage period. Microbiota found in mussels after depuration (day 0) had a different grouping of dominant phyla than did the mussels during storage.

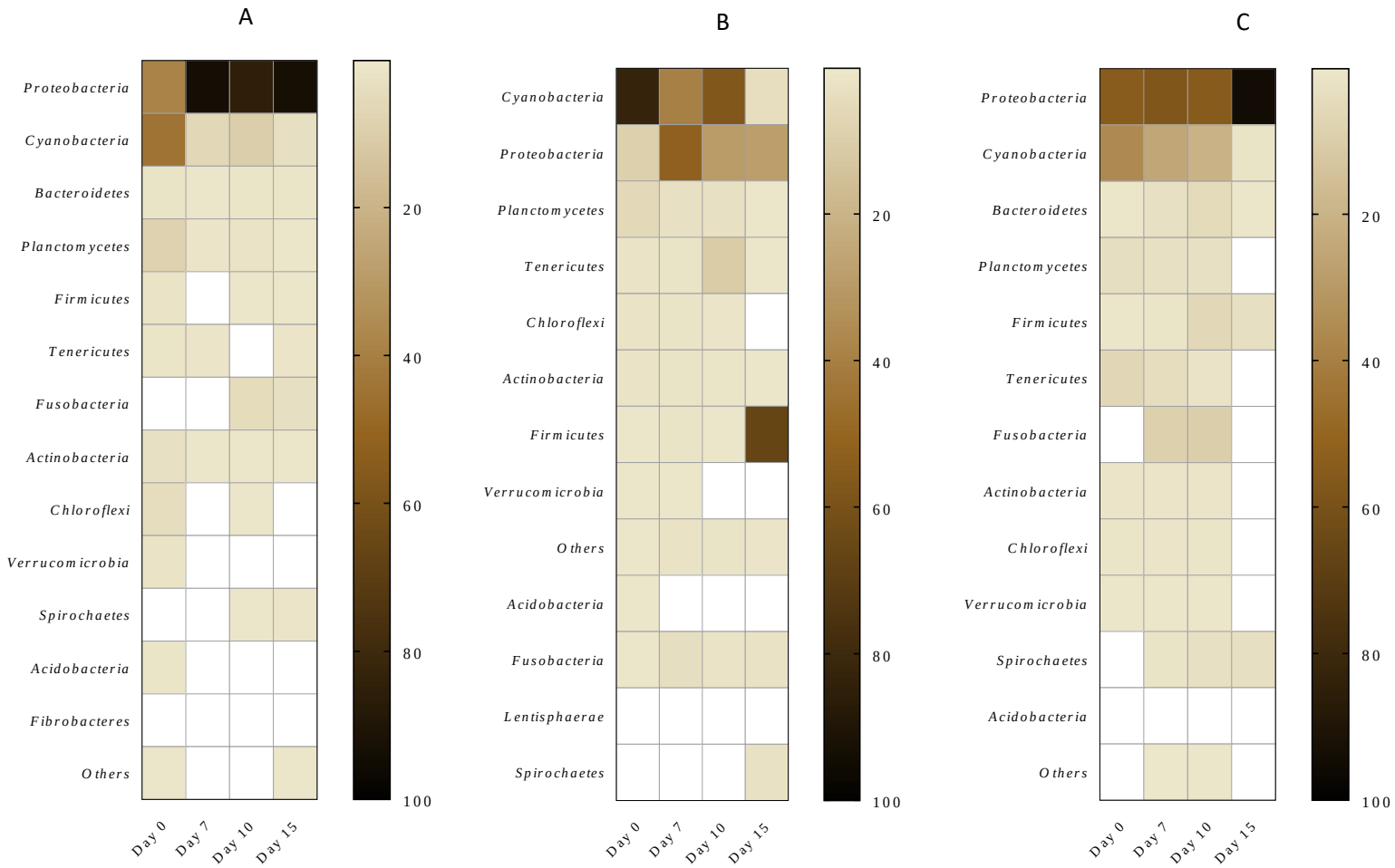


**Figure 4.8:** The heat-map (threshold ≥ 0.1%) of microbial community succession at phylum level observed in the mussel meat of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. The change in relative abundance (%) within the microbial community of each phylum is shown by colour intensity. White indicates extremely low (< 0.1%) abundance, yellow indicates very low (0.1- 10%), pale brown indicates moderate (10 – 49%), brown indicates (50% - 79%) and dark brown indicates high (80 - 100%) abundance while abundance > 50% is further termed dominant.

In the undepurated mussels, a total of 10 dominant phyla (here considered to be identified phyla > 0.1% relative abundance) comprising of Cyanobacteria, Proteobacteria, Planctomycetes, Tenericutes, Chloroflexi, Actinobacteria, Firmicutes, Verrucomicrobia, Acidobacteria, Lentisphaerae and Fusobacteria were observed on day 0. Among these, Cyanobacteria (82%) and Proteobacteria together accounted for 90.49% of the relative abundance. By day 7, the known phyla were reduced to 9 unlike 6 in undepurated mussels. Cyanobacteria (39%) and Proteobacteria (51%) contributing the most abundance. On day 10, Cyanobacteria (56%), Proteobacteria (28%) and Tenericutes (10%) became more dominant while on day 15, Proteobacteria (27.55%) and Firmicutes (65%) constituted the most abundant phyla. A total of 12 known phyla were observed in the mussel meat of commercially-depurated mussels namely: Proteobacteria, Cyanobacteria, Bacteroidetes, Planctomycetes, Firmicutes, Tenericutes, Fusobacteria, Actinobacteria, Chloroflexi, Verrucomicrobia, Spirochaetes and Acidobacteria (Figure 4.8C). Nine phyla were observed on day 0. Proteobacteria was most dominant (54%) on day 0 and across the storage period peaking at 94% on day 15. The relative abundance of Cyanobacteria was 35% on day 0 while Tenericutes was 6%. The same phyla (11) were observed on both days 7 and 10 and Proteobacteria remained the most dominant at 57% and 54% respectively. There was a decrease in relative abundance of Cyanobacteria on day 7 and 10 (to 24% and 20%) while Fusobacteria apparently increased to 8% and 9% relative abundance respectively. At the end of the storage, the number of dominant phyla observed on day 15 decreased to only 5 from 11 on days 7 and 10 compared to 9 phyla observed in undepurated and 7 in depurated on day 15.

Overall, more phyla were observed in undepurated mussels (13) than depurated mussels (11) and commercially-depurated mussels (12). Cyanobacteria was dominant at the start of storage in undepurated and depurated mussels. A complete shift in dominant phylum was observed on day 15. Proteobacteria became dominant in undepurated mussels and Firmicutes in depurated mussels. However, Proteobacteria remained increasingly dominant in commercially-depurated mussels from the start to the end of storage.

The heat-map below shows the change in microbial community structure in the mussel meat at the genus level during storage of live mussels as shown by the colour intensity of relative abundance (Figure 4.9). A diverse microbial community was observed in the undepurated mussel meat (Figure 4.9A). A total of 37 genera from the following 9 phyla were identified: Actinobacteria (3 genera), Bacteroidetes (4 genera), Cyanobacteria (1 genus), Firmicutes (4 genera), Fusobacteria (1 genus), Planctomycetes (1 genus), Proteobacteria (18 genera), Tenericutes (1 genus) and Verrucomicrobia (4 genera).

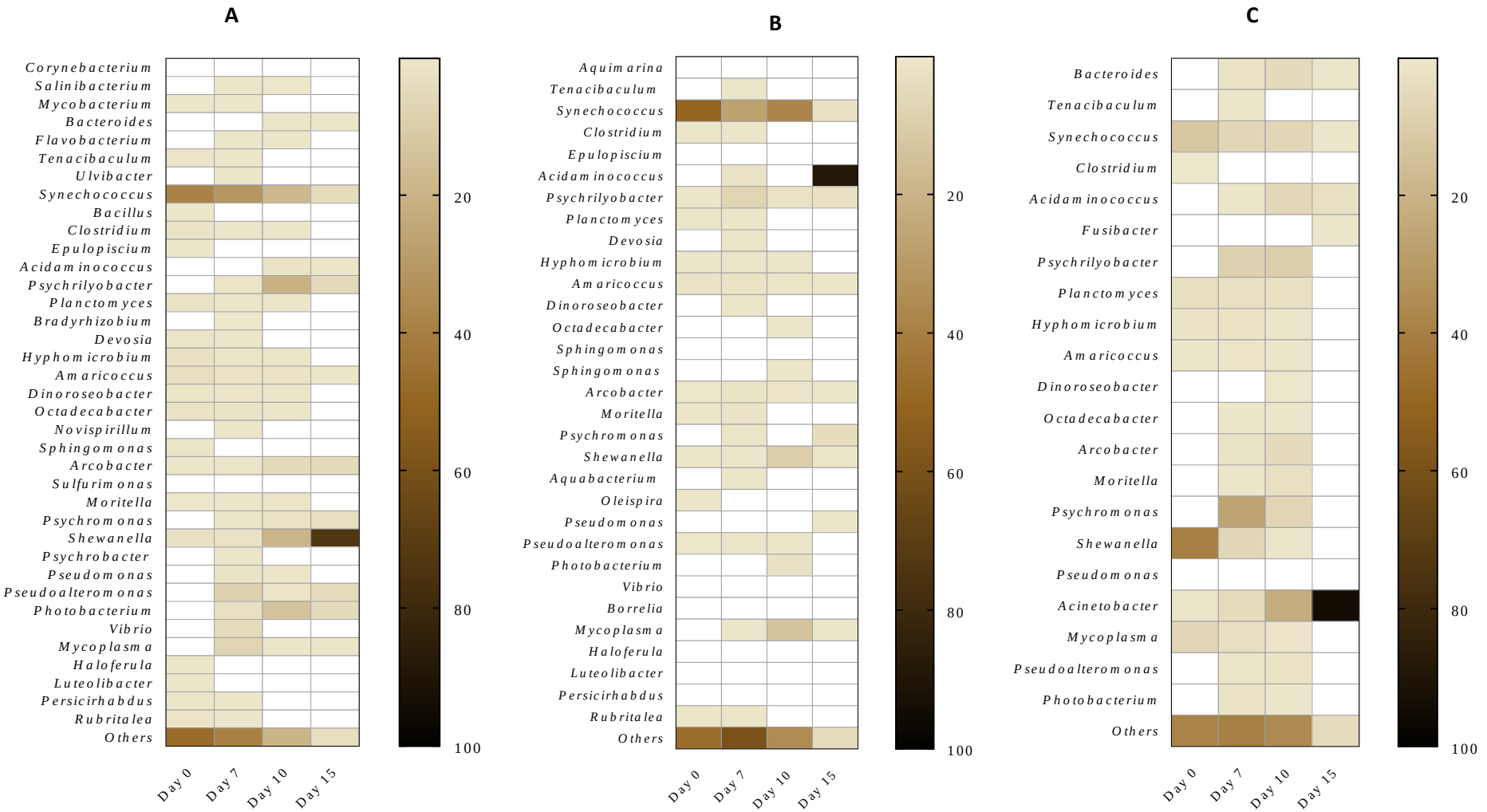


**Figure 4.9**: The heat-map (threshold ≥ 0.1%) of microbial community succession at genus level observed in the mussel meat of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. The change in relative abundance (%) within the microbial community of each phylum is shown by colour intensity. White indicates extremely low (< 0.1%) abundance, yellow indicates very low (0.1- 10%), pale brown indicates moderate (10 – 49%), brown indicates (50% - 79%) and dark brown indicates high (80 - 100%) abundance while abundance > 50% is further termed dominant.

Among these genera, 18 were from the phylum Proteobacteria as they contribute to the diversity of indigenous microbiota though often at low relative abundance. Twenty-eight known genera were observed on day 0 with *Synechococcus* being most dominant (39%) and remaining identified genera constituting no more than 3% relative abundance. Furthermore, 47% of genera present on day 0 were not identified. An apparent increase in the number of identified genera was observed on day 7 from 20 to 28. While some of the genera observed on day 0 were present on day 7, some were newly observed. Among the newly observed genera are *Salinibacterium, Flavobacterium, Psychromonas, Psychrobacter, Pseudomonas, Pseudoalteromonas, Photobacterium* and *Vibrio*. *Synechococcus* was still dominant on day 7 aside other unknown genera classified as "others" (39%). *Mycoplasma* (7%), (*Pseudoaltermonas* (8%), *Vibrio* (4%) and *Photobacterium* (3%) were observed. A reduction in the total known and dominant phyla in undepurated mussel meat was observed on day 10 with 20 genera observed. Only *Acidaminococcus* was the newly observed genus on day 10. While the relative abundance of *Synechococcus* decreased by almost half to 17%, *Psychrilyobacter, Shewanella* and *Photobacterium* apparently increased to 20%, 19% and 13% on day 10 from 1%, 2% and 2% on day 7. On day 15, *Shewanella* increased to 73% while *Synechococcus* reduced drastically to 4%. *Psychrilyobacter* and *Photobacterium* also reduced to 4%. Notably, the relative abundance of unidentified genera continued decreasing on days 10 and 15 to a final percentage of 3% suggesting dominance of a few spoilage bacteria that grow well in storage and the decline of indigenous microbiota.

The changes in microbial community composition of mussel meat of depurated mussels at genus level during storage are shown by the heat map in Figure 4.9B. A total of 30 known genera from 8 known phyla were observed in the mussel meat throughout the storage

period with Proteobacteria constituting the highest proportion (17/30) of the genera present as observed in undepurated mussels. Day 0 started with 12 genera that are associated with 7 different phyla: Cyanobacteria – 1 genus (*Synechococcus* – 50%); Verrucomicrobia – 1 genera (*Rubritalea* - 0.2%); Firmicutes – 1 genera (*Clostridium* - 0.2%); Fusobacteria – 1 genus (*Psychrilyobacter* - 0.2%); Planctomycetes - 1 genus (*Planctomyces* - 0.3%) and Proteobacteria – 7 genera (*Amaricocus* - 1.2%*, Shewanella* - 0.13%, *Oleispira* - 0.1%, *Moritella* - 0.1%, *Pseudoalteromonas* - 0.1%*, Arcobacter* - 0.1%, and *Hyphomicrobium* - 0.3%). A large proportion (47%) of genera were not identified.

The known dominant genera apparently increased from 12 on day 0 to 17 on day 7 from 8 phyla. Among these genera, 10 were previously observed on day 0. Similarly, 10 genera (6 from day 0 and 4 on day 7) were from the phylum Proteobacteria, indicating that Proteobacteria contribute significant diversity to the indigenous microbiota. *Synechococcus* (26%), *Acidaminococcus* (1%) and *Psychrilyobacter* (7*%*) were the dominant genera on day 7 and again there was 59% of unknown genera. On day 10, the known genera reduced to 11 among which 8 genera (*Psychrilyobacter, Hyphomicrobium, Amaricocus, Synechococcus, Arcobacter, Shewanella, Pseudoalteromonas* and *Mycoplasma*) were previously present on day 7 and 8 genera from the phylum Proteobacteria (*Hyphomicrobium, Amaricocus, Arcobacter, Shewanella, Pseudoalteromonas, Octadecabacter, Sphingomonas* and *Photobacterium*) were observed. The dominant genera were *Synechococcus* (37%), *Mycoplasma* (13%), *Shewanella* (9%), *Photobacterium* (2%) and *Psychrilyobacter* (2%) constituting 63% of all the known phyla while 35% were unknown. It was observed that on day 15, *Acidaminococcus* from the phylum Firmicutes became most dominant (88%) genus

which was a complete shift from days 0 - 10. Other dominant genera on day 15 are *Psychromonas* (3%), *Synechococcus* (2%) and *Psychrilyobacter* (2%).

Twenty-one known genera from 7 phyla (Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria and Tenericutes) were observed in the mussel meat of commercially-depurated mussels (Figure 4.9C). Although there were many unknown genera, only 8 genera were dominant on day 0 among which *Shewanella* (40%) was more abundant. *Synechococcus* (12%) was the next most abundant genus and then *Mycoplasma* (6%). However, on day 7, *Psychromonas* became more dominant (25%) while *Shewanella* decreased to 6% and Psychrilyobacter was 8%. The detectable microbial community became more diverse at both phylum and genus levels on day 10. The relative abundance of *Psychromonas* decreased to 6.30% on day 10 but *Acinetobacter* suddenly increased to 22% on day 10 from 4% on day 7. *Acinetobacter* was observed to sharply increase to 93% on day 15.

Mussel meat of undepurated mussels had more genera (38) than other treatments (30 genera in depurated and 27 genera in commercially-depurated mussels). *Synechococcus* was dominant at start of storage in both undepurated and depurated mussels. while *Shewanella* was dominant in commercially-depurated mussels. At the end of storage, *Shewanella* was dominant in undepurated, *Acidaminococcus* in depurated mussels and *Acinetobacter* in commercially-packed mussels which was not observed in other treatments signifying change in microbial diversity between the treatments.

In comparison, *Synechococcus* was observed to be changing in the meat of depurated and undepurated mussels. In both samples, there was a significant difference ($p < 0.05$) in the occurrence of *Synechococcus* between day 0 and 15. *Acidaminococcus* was significantly different ($p < 0.05$) on day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15 in depurated mussel meat. However, *Shewanella*, was significantly different ($p < 0.05$) on day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15 in undepurated mussel meat. In the meat samples of the commercially depurated mussels, *Acinetobacter* was significantly different ($p < 0.05$) on day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15.

### 4.3.4 Bacterial community diversity and succession in pouch water at phylum and genus levels

In the pouch water of undepurated mussels, 23 OTUs were observed on day 0, 9 on day 7, 12 on day 10 and 9 on day 15 (Table 4.4). A total of 5 OTUs on day 0, 4 on day 7, 10 on day 10 and 9 on day 15 at phylum level were observed in the pouch water of depurated mussels (Table 4.4). In the pouch water of commercially-depurated mussels, 15 phyla were observed on day 0, 18 on day 7, 17 on day 10 and 13 on day 15 (Table 4.4). The highest number of OTUs at genus level was observed on day 0 unlike day 15 in depurated and day 10 in commercially-depurated mussels. The least number of OTUs in undepurated mussels was on day 7, day 0 in both depurated and commercially-depurated mussels.

**Table 4.4:** The distribution of OTUs at different levels in the pouch water of undepurated mussels (A), depurated mussels (B) and commercially-depurated mussels (C) stored at 4°C for 15 days.

| Sample | Storage days | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|
| Undepurated | Day 0 | 23 | 62 | 116 | 197 | 284 |
| | Day 7 | 9 | 12 | 20 | 26 | 31 |
| | Day 10 | 12 | 18 | 25 | 37 | 52 |
| | Day 15 | 9 | 15 | 21 | 28 | 36 |
| Depurated | Day 0 | 5 | 9 | 16 | 23 | 26 |
| | Day 7 | 4 | 11 | 19 | 26 | 33 |
| | Day 10 | 10 | 17 | 26 | 36 | 44 |
| | Day 15 | 9 | 15 | 22 | 34 | 46 |
| Commercial | Day 0 | 15 | 19 | 32 | 44 | 57 |
| | Day 7 | 18 | 24 | 37 | 57 | 73 |
| | Day 10 | 17 | 24 | 40 | 58 | 78 |
| | Day 15 | 13 | 22 | 37 | 52 | 70 |

Four OTUs are shared in the pouch water of undepurated mussels across the sampling days (Figure 4.10A). There are 17 unique OTUs on day 0, but fewer on days 7 (2), 10 (5) and 15 (3). Only 1 OTU (Proteobacteria) was shared in depurated mussels throughout the sampling days at phylum level (Figure 4.10B) unlike 4 in undepurated mussels. The numbers of unique OTUs were higher on day 10 and day 15 than on days 0 and 7 (both 2 unique OTUs). In the pouch water of commercially-depurated mussels, 2 OTUs were shared in all the sampling days (Figure 4.10C) almost similar to depurated (1 OTU) but different from undepurated (4 OTUs) mussels. Six unique OTUs were observed on day 0, 11 on day 7, 10 on day 10 and 7 on day 15. The OTUs obtained at other levels are summarised in Table 4.4 and clearly there are far fewer OTUs in the pouch water than in the mussel meat. Overall, the number of OTUs in mussel meat were more than those in pouch water.

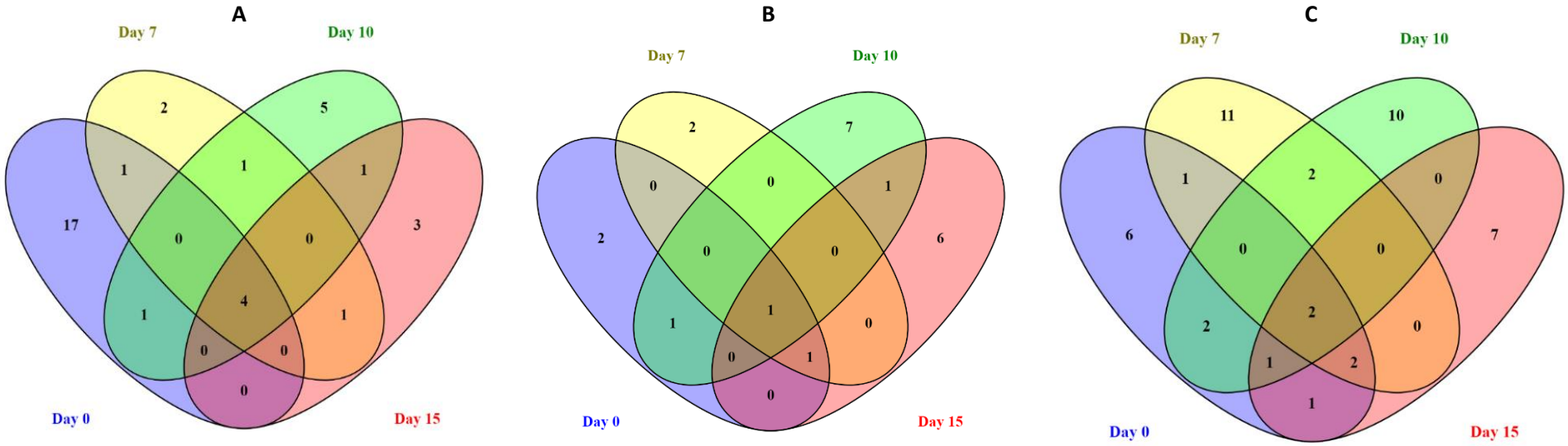


**Figure 4.10:** Venn diagram showing the distribution (97% similarity) of the OTUs at phylum level present in the pouch water samples of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. Each number indicates shared or unique OTUs in the samples in each sampling day. Different colour indicates different sampling days (day 0 = blue, day 7 = yellow, day 10 = green and day 15 = pink).

Across the storage time, 18 known phyla were observed in the pouch water of undepurated mussels (Figure 4.11A). However, only 11 phyla were prevalent on day 0 among which Proteobacteria was most dominant (79%) and Cyanobacteria was 11.22% of the overall phyla. There was increase in the relative abundance of Proteobacteria on day 7 and 10 to 99%. Bacteroidetes was just 0.45% on day 10. On day 15, while Proteobacteria was still most dominant (79%), there was decrease in the abundance and Firmicutes become prominent (20%) aside other Spirochaetes (1%) and Fusobacteria (0.1%) observed. The relative abundance of known phyla present in the pouch water of depurated mussels represents 98% of all the phyla (Figure 4.11B). A total of 4 dominant phyla comprising of Cyanobacteria, Proteobacteria, Tenericutes and Firmicutes were observed on day 0. This was less than the total known phyla observed in the undepurated mussels.

Proteobacteria had by far the highest relative abundance on days 0 – 10 (> 95%) while Cyanobacteria represented only 2% on day 0 and then were < 0.1% thereafter. On day 10 Fusobacteria were the second most abundant phylum at 1.4%. Apparent increase in the dominant phyla from 2 on day 10 to 5 on day 15 was observed. Between days 10 and 15, there was a complete change in dominance in the microbiota as Firmicutes rose to 98% relative abundance and Proteobacteria declined to < 1%. This was also like the observation on day 15 in mussel meat.

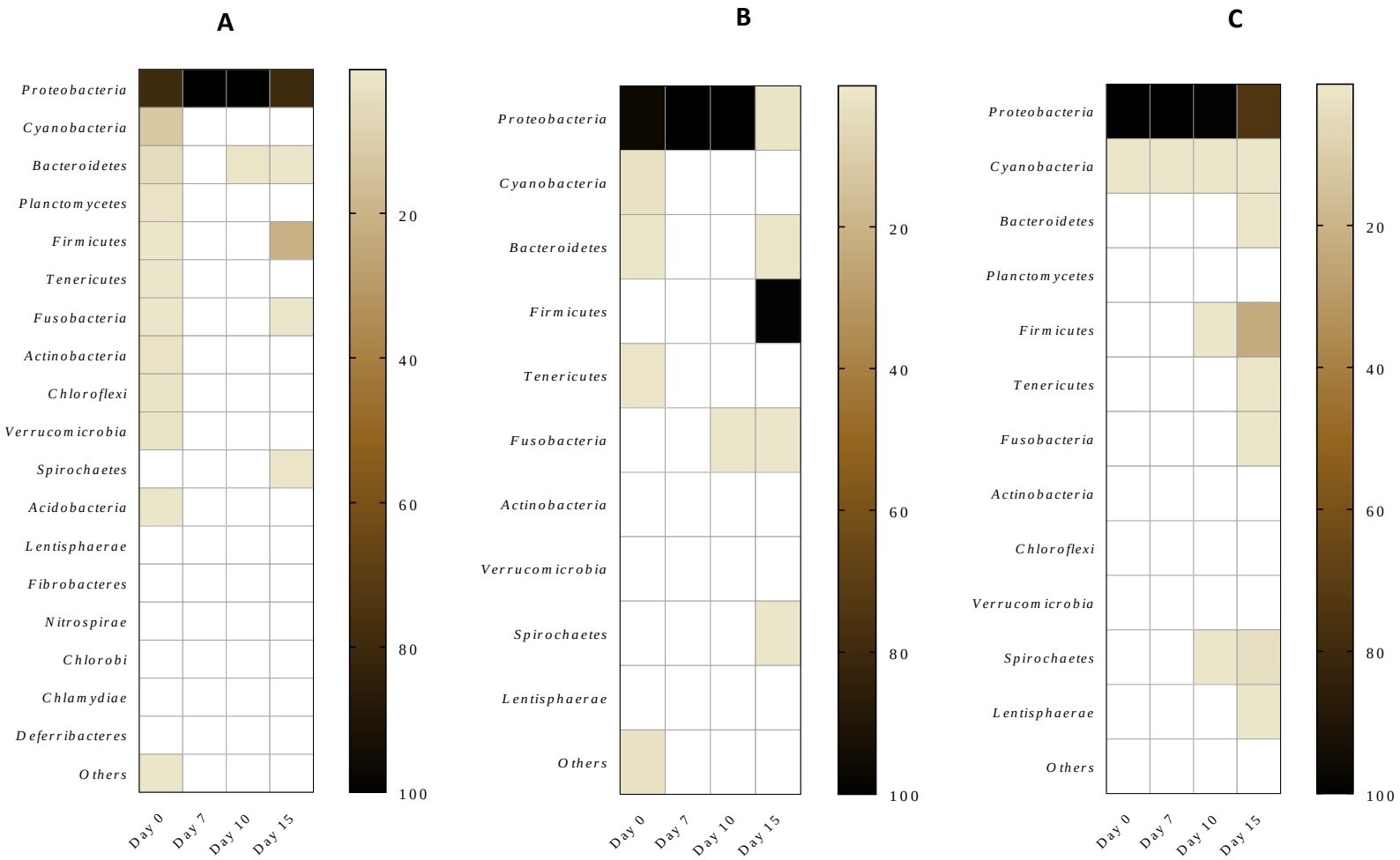


**Figure 4.11**: The heat-map (threshold ≥ 0.1%) of microbial community succession at phylum level observed in the pouch water of of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. The change in microbial community relative to the abundance (%) of each phylum is shown by colour intensity. White indicates extremely low (< 0.1%) abundance, beige indicates very low (0.1- 10%), tan/light brown indicates moderate (10 – 49%), brown indicates (50% - 79%) abundant and dark brown indicates high (80 - 100%) abundance while abundance > 50% is further termed dominant.

Twelve phyla were observed to be dominant in pouch water of commercially-depurated mussels across the storage days (Figure 4.11C). Only two phyla were dominant on day 0 and 7. However, more phyla were observed on days 10 and 15. Proteobacteria was more dominant (99%) on day 0, day 7 (99%), day 10 (98%) and 73% on day 15. Firmicutes suddenly increased from 0.4% on day 10 to 23% on day 15 thereby compensating for the decrease in the relative abundance of Proteobacteria.

A total of 31 known genera from 6 genera were observed in the pouch water of undepurated mussels among which 25 were dominant on day 0 (Figure 4.12A). *Acinetobacter* which is a genus from the phylum Proteobacteria was the most dominant (55% relative abundance). There was sharp decrease in the total genera on day 7 as only 2 known genera were observed to be dominant (*Acinetobacter* – 99% and *Pseudoalteromonas* – 0.6%). On day 10 only 5 genera were dominant. The relative abundance of *Acinetobacter* decreased on day 10 (76%) and day 15 (51%) while *Sulfurospirillum* increased from 5% on day 10 to 19% on day 15. Other genera observed on day 15 were *Shewanella* (8% and *Fusibacter* (20%). As in the communities of undepurated mussel meat, the unknown genera represented a small proportion of the relative abundance of genera, particularly after day 0.

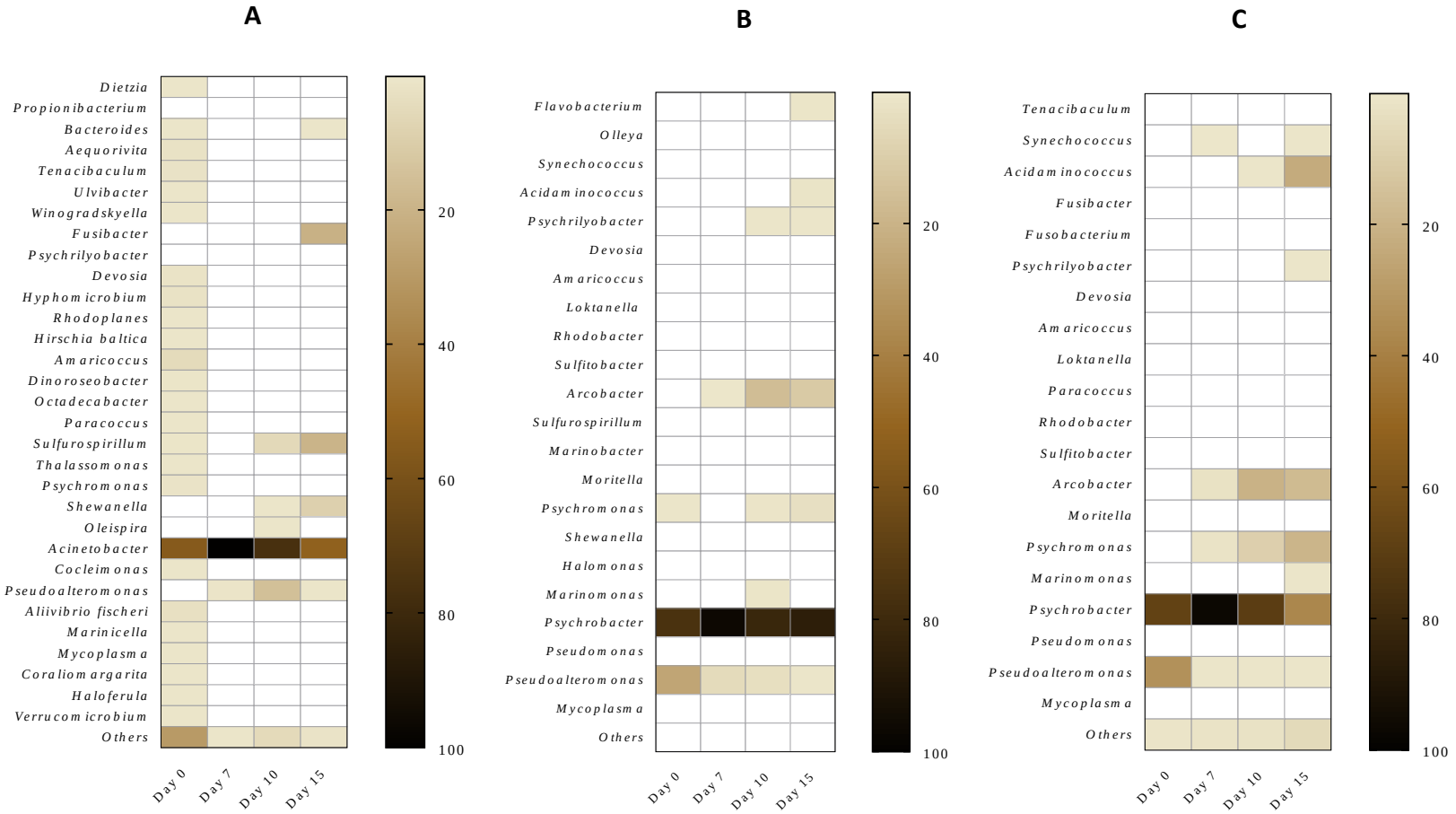


**Figure 4.12**: The heat-map (threshold ≥ 0.1%) of microbial community succession at genus level observed in the pouch water of of undepurated (A), depurated (B) and commercially-depurated mussels (C) stored at 4°C for 15 days. The change in microbial community relative to the abundance (%) of each genus is shown by colour intensity. White indicates extremely low (< 0.1%) abundance, beige indicates very low (0.1- 10%), tan/light brown indicates moderate (10 – 49%), brown indicates (50% - 79%) abundant and dark brown indicates high (80 - 100%) abundance while abundance > 50% is further termed dominant.

A total of 22 known genera from 7 phyla were observed in the pouch water of depurated mussels (Figure 4.12B). Only 3 genera comprising of *Psychrobacter* (75%), *Pseudoalteromonas* (25%) and *Psychromonas* (0.4%) from the phylum Proteobacteria dominated on day 0. This was different from the observation in undepurated mussels. While 3 genera were also observed on day 7, there was a change in one of the phyla. *Arcobacter* replaced *Psychromonas* and *Psychrobacter* (96%) increased by 21%. The number of dominant genera doubled on day 10 with *Psychrobacter* still dominant (80%) and *Arcobacter* increased to 11%. Like undepurated mussels, only one genus was dominant although the genus observed was different (*Psychrobacter*). Both *Marinomonas* (0.4%) and *Psychrilyobacter* (0.2%) were < 0.1 in the pouch water on day 10 while *Arcobacter* increased to over 15%. On day 15, the dominant genera apparently increased to 7. *Acidaminococcus* (1%) and *Flavobacterium* (0.4%) were newly observed.

In the pouch water of commercial mussels, 20 known genera were observed (Figure 4.12C). Two genera from the phylum Proteobacteria, namely *Psychrobacter* and *Pseudoalteromonas*, were more dominant on day 0 (67% and 33%). However, *Psychrobacter* significantly increased on day 7 to 96% but decreased on day 10 to 69%. On day 10, there was increase in the abundance of both *Arcobacter* by 20% and *Psychrobacter* by 9%). On day 15, a sudden increase in *Acidaminococcus* (23%) from 0.4% on day 10 was observed. in terms of the dominant genera, there was a more diverse microbial community on day 15 than other days and *Psychrobacter* was still the most dominant (37%) genus. As observed in the relative abundance of phyla, the pouch water of undepurated mussels had more known genera (31) than the other treatments. *Psychrobacter* was dominant throughout the storage

days in all the treatments. However, its relative abundance was more in undepurated mussels (75%), 67% in depurated and least in commercially-depurated mussels (37%).

In the pouch water of depurated mussels*, Psychrobacter* was significantly different ($p < 0.05$) on day 0 vs. day 7, day 7 vs. day 10 and *Pseudoalteromonas* was significantly different ($p < 0.05$) on day 0 vs. day 7, day 0 vs. day 10, day 0 vs. day 15. Similarly, *Arcobacter* was significantly different ($p < 0.05$) on day 0 vs. day 10, day 7 vs. day 10. Only *Acinetobacter* was significantly different ($p < 0.05$) on day 0 vs. day 7, day 7 vs. day 10, day 7 vs. day 15, day 10 vs. day 15 in the pouch water of undepurated mussels. In the pouch water, *Psychrobacter* was significantly different ($p < 0.05$) on day 0 vs. day 7, day 0 vs. day 15, day 7 vs. day 15, day 10 vs. day 15 while *Pseudoalteromonas* day 0 vs. day 7, day 0 vs. day 10, day 0 vs. day 15.

## 4.4 Discussion

In Chapter 3 of this thesis, it was suggested there is need to understand the effect of depuration on spoilage microbial community and succession because more than one volatile metabolite was used to assess the freshness and spoilage of MAP live mussels. This Chapter therefore aimed to describe the microbial communities in MAP live mussels packed with 80% oxygen and stored at 4°C for 15 days. It sought to describe the impact of depuration procedures on the microbiota present in live mussels during storage relate this to mechanisms of mussel spoilage. The microbial diversity observed in this study could have been affected by the many factors including the initial treatments of depuration. Depuration reduces microbial load as demonstrated by the results of total viable count (TVC) obtained in Chapter 3. The commercial packs were depurated for only 2 hr compared to 8 hr depuration of laboratory packs. Additionally, the commercially packs had more mussels than other treatments, which could engender further increases in observed diversity and richness from inter-animal variability. Huge changes in the microbial community present in the mussel meat of depurated mussels stored at 4°C for 15 days were observed at both phylum and genus levels. The initial indigenous microbiota was diverse though dominated by a small proportion of taxa. The dominant (organisms with at least 50% abundance) taxa changed radically over time, suggesting that storage conditions are radically different from the normal environment of mussels. The longer the storage, the less diverse were the dominant taxa. The wide diversity at the phylum level observed in this current study reflects the marine environment in which the mussels were harvested. The increase in the total microbial diversity with time could not be from external sources since it is a closed system. The observed increase was possibly because of the suitability of intrinsic conditions such as

nutrient availability because of the death of mussels and slurry in the pouch water. Similarly, changes in diversity could be driven by relative abundance/dominance of different groups which was observed in this study. The gradual reduction of the phyla from highly abundant to extremely low abundance shows the progression of succession of the microbial community.

Trabal et al. (2012) studied the microbial community in depurated oysters using V3–V5 regions of the 16S rRNA gene. They obtained 13 phyla and over 200 genera. However, Proteobacteria was the most dominant phylum with variations in the dominant genera. Chen et al. (2017) studied the bacterial community profiling of gills of MAP oysters stored at 4°C based on the analysis of V3 region of 16S rRNA gene using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). They reported that both *Lactobacillus* and *Lactococcus* were dominant bacteria. Temporal Temperature Gel Electrophoresis (TTGE) was used to microbial community in MAP scallop meat (*Pecten maximus*) stored at 4°C for 8 days (Coton et al., 2013). *Shewanella*, *Pseudomonas* and *Brochothrix* were reported in their study as dominant bacteria at the end of storage.

The shift in the phyla present in the mussel meat observed in this current study was like the observation of Fernandez-Piquer et al. (2012) who reported shifts from Spirochaetes, Proteobacteria, Planctomycetes, Verrucomicrobia and Cyanobacteria to Fusobacteria during storage of live oysters. They reported that *Psychrilyobacter* which is a genus of the phylum Fusobacteria dominated (44%) live oysters stored at 4°C and the overall microbial

community diversity in oysters indicated the influence of storage temperature at postharvest storage conditions.

Huge change in microbial community richness and diversity was observed in the mussel meat of undepurated and depurated mussels at the onset of the storage. The fewer phyla observed in the pouch water on day 0 was because sterile freshwater was added to the pouch and the only source of bacteria in the pouch water was from the surface microbiota and gut contents of mussels.

At the start of the storage (day 0) of the samples, Cyanobacteria and Proteobacteria were dominant phyla present in the mussel meat and pouch water of the treatments and control. Cyanobacteria was found to be dominant in the mussel meat of both undepurated and depurated mussels with *Synechococcus* as the dominant genus from day 0 – 7 (undepurated) and day 0 – 10 (depurated) and was not dominant in commercially packed mussels. This could indicate the fact that *Synechococcus* were from the marine environment either in the gut or on the external surfaces of the mussels. *Synechococcus* constitutes a large portion of mussels' diet, which is supported by the fact that their relative abundance declines with storage – they aren't growing in or on mussels during storage.

*Acidaminococcus* was dominant on day 10 in the mussel meat of undepurated mussels and day 15 in depurated mussels. *Acidaminococcus*, members of the Firmicutes, are Gram-negative, non-motile, anaerobic diplococci that utilise amino acids as sole energy sources.

They are mostly found in gastrointestinal tracts (GIT) of seafood and are oxidase and catalase-negative (Chang et al., 2010; Jumas-Bilak et al., 2007; Russell, 2015). The use of amino acids suggests that *Acidaminococcus* is associated with the presence of readily available amino acids and the most likely source of these would be from mussel protein degradation. This suggests that *Acidaminococcus* are associated with spoiled mussels.

*Shewanella* was dominant on day 15 in the mussel meat of undepurated mussels and day 0 in commercially packed mussels. *Shewanella* is a genus comprising of Gram-negative, oxidase positive, rod shaped and catalase positive bacteria that cannot ferment glucose and are present in the marine environment. Members of this genus such as *S. baltica* have been associated with spoilage of seafood such as fish (Ge et al., 2017), shrimp (Macé et al., 2013a) and oyster (Madigan et al., 2014) and are evident with the production of $H_2S$ or spoilage volatile metabolites such as dimethyl disulphide in fish (Parlapani et al., 2017). *Shewanella* have been reported to be able to use anaerobic respiration using electron acceptors such as trimethyl-amine-N-oxide (TMAO) in place of oxygen and could survive in oxic/anoxic conditions (Brettar et al., 2002; Venkateswaran et al., 1999). Such oxic – anoxic conditions may be found in MAP especially towards the end of the storage (day 10 onwards) as the packs have stagnant water in the pouch. This suggests a reason why *Shewanella* were more dominant in mussel meat rather than the pouch water. It should be noted that *Shewanella* species such as *S. baltica* are motile and could produce biofilms (attachment to surfaces) (Zhao et al., 2016). Similarly, because mussels like other seafood are rich in cysteine this enhances growth of *Shewanella* because of its biochemical actions of producing of $H_2S$ from cysteine. It can also produce methylmercaptane ($CH_3SH$) and dimethylsuphide - $(CH_3)_2S$ from amino acids such as methionine (Lopez-Caballero et al., 2001).

*Acinetobacter* was observed to be dominant throughout the storage period in the pouch water of undepurated mussels. *Acinetobacter* is a genus of Gram-negative, non-fermenter, diplococci, non-motile, non-pigmented, aerobic, oxidase-negative and catalase-positive bacteria that are found in aquatic environment (Bouvet and Grimont, 1986; Clemmer et al., 2011; Kozinska et al., 2014). At early stationary phase, *Acinetobacter* produce enzymes such as lipase and are capable of producing multiple quorum sensing (QS) signalling molecules (Gonzalez et al., 2001). QS signalling molecules mediate spoilage gene expression in spoilage bacteria at increased population density (Gu et al., 2013).

Members of the genus *Acinetobacter* have been isolated from various seafood such as the skin and flesh of Turbot (*Scophthalmus maximus*) that was stored at 4°C (Zhang et al., 2016), tropical red drum (*Sciaenops ocellatus*) stored at 4°C (Silbande et al., 2018) and sea bream stored at 4-5°C (Parlapani et al., 2013b). However, the presence of *Acinetobacter* in mussels has not been previously reported. Recently, it was reported that although *Acinetobacter* has low spoilage potential, it enhances the growth of other spoilage bacteria by means of quorum sensing (QS) signalling molecules such as N-acyl homoserine lactones (AHLs). Zhang et al. (2016) reported that when total viable count (TVC) reaches 8 log CFU $g^{-1}$, or *Enterobacteriaceae* reached 6 log CFU $g^{-1}$, QS signalling molecules are triggered in spoilage bacteria present thereby causing spoilage of the fish at cold storage. Hence, this shows that the presence of *Acinetobacter* in mussel meat of commercially-depurated mussels (depurated for two hours) and pouch water of undepurated mussels could enhance spoilage of mussels by other bacteria, especially *Shewanella* identified in this study.

*Psychrobacter was* dominant in the pouch water of depurated and commercially-depurated mussels throughout the storage period. *Psychrobacter* are non-motile, psychrotolerant or psychrophilic, oxidase-positive, non-pigmented, Gram-negative rod or coccobacilli that can survive at low temperatures(Bozal et al., 2003) (Ayala-del-Río et al., 2010; Bozal et al., 2003; Juni and Heym, 1986). *Psychrobacter* have been isolated from marine environment, the flesh and gut content of seafood such as fish, oyster and lobster (Bakermans et al., 2006; Romanenko et al., 2004). *Psychrobacter* adapt and survive in fatty acid-rich environments which explains why they dominate in the pouch water especially on day 15 as the mussels have spoiled, degraded and the pouch water became a slurry. *Psychrobacter* can breakdown lipids and also hydrolyse amino acids resulting in off-odours such as trimethylamine - TMA (Broekaert et al., 2013). *Psychrobacter* has been identified as spoilage bacteria in other seafood such as brown shrimp stored at 4°C for 9 days (Broekaert et al., 2013). This spoilage and lipolytic potential of *Psychrobacter* could contribute to the objectionable smell observed from day 10 when the packs where opened. However, in this current study, *Psychrobacter* was not observed in the mussel meat of depurated mussels. This implies that *Psychrobacter* survived in aquatic environment of the pouch water or if presence on the mussels, which means it is a transient organism that can be easily removed by depuration.

The significant impact of depuration on the microbial community in live mussels and the contribution of the microbial community to the spoilage mechanism was demonstrated in this study. The dominance of Cyanobacteria only on day 0 and the shift to Proteobacteria from days 7 to 15 and the dominance of Cyanobacteria in undepurated mussels on days 0 – 10 indicated that depuration only removes most of the microbes that are loosely attached

to the mussels. At the genus level, undepurated and depurated mussels started with the dominance of *Synechococcus* till day 7 in undepurated day 10 in depurated. A shift to *Acidaminococcus* on day 10 was noticed in undepurated mussels. This was not observed in depurated mussels. On day 15, change in dominant taxon was noted in both undepurated and depurated. While *Shewanella* became dominant in undepurated, *Acidaminococcus* was observed in depurated mussels. In comparison, commercially packed mussels had changes in the microbial community twice between day 0 and day 10 like undepurated mussels. *Shewanella* that was observed on day 0 in commercially packed mussels may have aided the growth of *Psychrobacter* in the packs on day 7 because Shewanella produces signalling molecules that aid growth of other bacteria. *Acinetobacter* became dominant on both days 10 and 15. This indicated the mutualistic and synergistic contributions of these spoilage bacteria, which could contribute stress to the mussels. Undepurated mussels may have been stressed earlier as change in microbial community was first noticed on day 7 like that of commercially packed mussels. This may have affected the health of the mussels and contributed to mortality in addition to the environmental conditions in the packs and microbial exacerbation (TVC > 6 log CFU $g^{-1}$) that was more obvious between day 7 and 10 in undepurated mussels and commercially-depurated mussels as reported in Chapter 3 (Figures 3.3 and 3.7). The observed shift in the taxa on day 15 across the treatments indicated the synergistic contribution of other spoilage bacteria. For example, amino acids required by *Acidaminococcus* may have been readily available on day 10 in undepurated and day 15 in depurated mussels. At the end of storage (day 15), a different taxon was observed to be dominant in the treatments.

*Acinetobacter* in the pouch water of undepurated mussels could have provided a synergistic effect on the dominance of *Acidaminococcus* on day 10 in the mussel meat. *Psychrobacter* potentially provided a similar effect in depurated and commercially-depurated mussels. This implies that pouch water contributes significant spoilage to the mussel meat, especially in undepurated and commercially-depurated mussels because the later only had short (1-2 hr) duration of depuration. Therefore, lack (no depuration) and inefficient (short time depuration of 1-2 hr) depuration contributes to the faster rate of spoilage compared with effective depuration duration (8 hr). The mean TVC in pouch water was more than that of mussel meat. This may have exacerbated hypoxic areas and influenced the microbial community by favouring growth and dominance of Proteobacteria that are aerobic or facultative and readily consume oxygen. As hypoxic areas occur, more anaerobes like *Acidaminococcus* become dominant.

## 4.5 Conclusion

This study demonstrated the impact of depuration on the microbiota and the spoilage mechanism. As the consumption of oxygen increases, this resulted in hypoxic conditions towards the end of storage and contributed to the change in microbial community as indicated by dominance of aerobic or facultative bacteria (Proteobacteria). *Shewanella* was easily removed through depuration. However, spoilage bacteria such as *Acidaminococcus* were not easily removed although they are not important at the beginning but grew at the end during storage. Pouch water contributed a suitable biological medium for the growth of *Acinetobacter* and *Psychrobacter* and both enhanced the growth of spoilage bacteria such

as *Shewanella* and *Acidaminococcus* which could be through the production of QS signalling molecules. Effective depuration will therefore help in reducing spoilage bacteria and thus the rate of spoilage of live mussels. However, there is a need to study the spoilage potential of either axenic or mixed cultures of these spoilage bacteria. The detection limits of the methods and the small number of samples served as limitations for this study. The V1- V3 hypervariable gene used could serve as limitation because all the phyla may not be detected. Hence, other regions such as V3-4, and V4 region could be targeted in future studies as this could reveal significant differences in OTU abundances. Therefore, further studies with samples of multiple packs for reproducibility of the patterns and use of other sequencing methods that could be less expensive to understand the microbial diversity are recommended.

# Chapter 5: Assessment of spoilage potential and headspace volatile metabolites of hydrogen sulphide - producing bacteria isolated from modified atmosphere packaged live mussels

## 5.1 Introduction

Seafood is reported to be high in quality protein, omega-3 fatty acids, essential micronutrients and minerals (Goulas et al., 2005). Contamination of seafood may occur in various stages such as processing, storage and distribution (Luu et al., 2017). Sources of contamination include water, facilities, equipment and handlers (Elbashir et al., 2018). The processing stage is particularly important, due to potential high microbial load on the contact surfaces (Mizan et al., 2015). The number and type of microorganisms present in the product determine whether the contamination will cause microbial deterioration or disease (Zwietering et al., 1991). The rate of spoilage of seafood is affected by both intrinsic and extrinsic factors such as composition of the seafood, water activity, packaging method and storage temperature.

Understanding the spoilage mechanism will help to maintain the quality and extend the shelf-life of seafood. While seafood indigenous enzymes are responsible for autolysis, microbial activities of specific spoilage organisms result in spoilage of seafood more often than chemical oxidation or autolysis (Parlapani et al., 2014). Although diverse microbiota is observed in seafood, however, few of these bacteria, known as specific spoilage organisms (SSO), cause spoilage (Boziaris and Parlapani, 2017).

SSO are off-flavour and off-odour metabolite-producing microbes that can dominate seafood (Olafsdóttir et al., 1997). These spoilage organisms are mainly Gram-negative bacteria (Boziaris and Parlapani, 2017). SSO produce off-odours like hydrogen sulphide, reduce trimethylamine n-oxide (TMAO) to trimethylamine (TMA) and biogenic amines due

to amino acid decarboxylation activities (Ge et al., 2017). They also produce enzymes such as DNase, lipase and protease (Muzaddadi et al., 2016). These spoilage markers reduce the palatability, shelf-life and consequently consumer acceptance of seafood (Kuuliala et al., 2018). Off-odour metabolites produced are collectively known as volatile organic compounds (VOC) and are broadly grouped into sulphur compounds, alcohols, amines, aldehydes, esters and organic acids and ketones often produced during seafood spoilage (Parlapani et al., 2017). VOC used as seafood spoilage indicators are characterised by an initial absence or presence in low levels in seafood and are mainly produced by spoilage bacteria which increase over the storage period (Parlapani and Boziaris, 2016). It is however, important to monitor VOC profiles during the whole storage period and record the order of appearance from first day of storage to end of shelf-life to associate particular compounds with fresh versus spoilt produce (Parlapani et al., 2014).

The spoilage potential of SSO and the production of VOC in other seafood such as shrimp (Mace et al., 2014) and fish (Parlapani et al., 2017) have been studied. Mace et al. (2014) reported *Shewanella baltica* as major spoilage bacterial isolate present in shrimp with the production of 14 spoilage VOC such as butanol, acetic and methyl mercaptan. Parlapani et al. (2017) also reported the production of dimethyl disulphide in fish inoculated with *Shewanella.* Similarly, *Shewanella* was one of the major spoilage organisms identified in Chapter 4 of this thesis. This current Chapter therefore focuses on evaluation of the spoilage potential of *S. baltica* isolated from modified atmosphere packaged (MAP) live mussels. To the best of my knowledge, no studies investigating the spoilage potential and VOC produced by spoilage bacteria isolated from MAP live mussels have been carried out. This study

therefore aimed to assess spoilage potential and headspace VOC of hydrogen sulphide ($H_2S$) - producing bacteria isolated from MAP live mussels. The results of this study will help to ascertain if, in addition to $H_2S$, whether microbial VOC lead to objectionable smell in MAP live mussels thereby expanding knowledge of the spoilage mechanism of this product.

## 5.2. Materials and methods

### 5.2.1 Mussel samples

Samples of freshly harvested mussels were obtained from the east coast of Tasmania, Australia as stated in Chapter 2. Samples were transported into the laboratory for processing in iced-packed Styrofoam boxes. Mussels were sorted, cleaned and depurated for 4, 8 or 12 hr in filtered seawater obtained from Low Head (George Town, Tasmania ) as described in Chapter 2, 3 and 4. Thereafter, 6 - 8 mussels were packed with 80% $0_2$ and 20% $N_2$ as described in the previous Chapters while 50 mL sterile fresh water was added to provide humid condition for the mussels. Commercially-depurated mussels (CM) were used as reference because this Chapter was directly linked to Chapters 3 and 4. Samples were stored at 4$^{o}$C for 15 days.

### 5.2.2 Microbiological analysis

Microbiological analysis was carried out on day 0, 4, 7, 10 and 15. On each sampling day, 10 g of mussel meat and pouch water was added separately to 90 mL of alkaline peptone water (ratio 1:10). The alkaline peptone water was prepared from bacteriological peptone 10 g (LP003T, Oxoid) and 20 g NaCl (Code 0314B92, Amresco, USA) with 1000 mL of distilled water and autoclaved at 121°C for 15 minutes (Dabade et al., 2015). This was then homogenized in a stomacher for 1 minute before serially diluting 1 mL of the homogenate in 9 mL of 0.1% alkaline peptone water. Thereafter, 10 µL of decimal dilutions ($10^0$ – $10^5$) from each mussel sample and pouch water was spread on Iron agar (IA) with the following composition: bacteriological peptone 20 g (Code LP0037, Oxoid, UK), Lab lemco powder 3 g (Code L29,

Oxoid, UK), yeast extract 3 g (Code LP0021, Oxoid, UK), ferric citrate 0.3 g, (Code 28381, BDH, UK), sodium thiosulphate 0.3 g (Code 517, Ajax Chemicals, Australia), cysteine 0.6 g (Code 37055, BDH, UK), NaCl 5 g (Code 0314B92, Amresco, USA) and agar 15 g (Code LP0011, Oxoid, UK) in 1000 mL of distilled water for the isolation of $H_2S$-producing bacteria (Gram et al., 1987; Parlapani et al., 2014). A categorical scale (- = no observed growth; + = barely present, a few colonies < 20; ++ = slightly present > 20 < 50 colonies; +++ = moderately present > 50 colonies; ++++ = abundantly present – too numerous to count - TNTC) was used to indicate the occurrence of isolates. A total of 46 black-pigmented colonies on Iron agar were selected randomly and their morphologies observed as described by Parlapani et al. (2015). Isolates were further purified on Tryptone soy agar (TSA) (Code CM0131, Oxoid, UK) before re-culturing on Iron agar to confirm production of black pigment due to precipitation of Iron sulphide (FeS) (Parlapani et al., 2015). Stock cultures were prepared from the isolates and preserved at - 80°C.

### 5.2.3 Biochemical identification

Selected biochemical tests as described in Bergey's manual (Kersters and Vancanneyt, 2005) and by Beaz-Hidalgo et al. (2015), Dehaut et al. (2014), Deng et al. (2014) and Fonnesbech et al. (2005) were used for phenotypic characterization of $H_2S$-producing isolates. Stock cultures of the isolates were sub-cultured from IA unto freshly prepared TSA and incubated at 25$^{o}$C overnight. Isolates were Gram stained, tested for catalase and oxidase reactions. Thereafter, isolates were cultured on IA to reconfirm iron precipitation. Similarly, isolates were cultured on DNase agar (Code CM0243, Oxoid, UK), Kligler iron agar (Code CM0033, Oxoid, UK), motility agar (Code CM43532, Oxoid, UK), and lipid agar (Code CM5378, Oxoid,

UK). API 20 E (Oxoid, England) and Microbact 24E (Oxoid, England) were also used for biochemical identification following manufacturer's instruction. Salt tolerance (0 - 6%) and growth at varying temperatures (4 - 37$^{o}$C) were also investigated.

## 5.3 Physiological characterization

### 5.3.1 $H_2S$-production on sulphur containing agar

Isolates were screened for the production of $H_2S$ on Iron agar (IA) with three different sulphur sources prepared as follows: $IA_1$ with the above components (section 5.2.2): $IA_2$ (only cysteine added), $IA_3$ (only sodium thiosulphate added) and Kligler Iron agar- KIA (Code CM0033, Oxoid). Isolates were then inoculated on freshly prepared sterile agar ($IA_1$, $IA_2$, $IA_3$ and KIA) and incubated at 25°C for 24 hr. The production of $H_2S$ on the agars after 24 hr incubation was designated positive (Parlapani et al., 2014).

### 5.3.2 Salt tolerance

Salt tolerance among the isolates was investigated. The following concentrations of salt (NaCl) were added to Iron agar: 0, 1.5%, 3% and 6% before autoclaving at 121°C for 15 minutes. After cooling the agar to 45°C, the molten agar was poured into plates (sterile Petri dishes) and allowed to solidify. Thereafter, 24 hr cultures of the isolates were inoculated into each of the plates and incubated at 25°C for 24 – 72 hr. Plates were observed on each day for visible growth and colony formation. The isolates that could not grow after 72 hr were regarded as not able to grow.

### 5.3.3 Ability to grow at different temperatures

The isolates were tested for ability to grow at different temperatures using TSA agar. Fresh cultures (18 – 24 hr) of the isolates were inoculated into freshly prepared TSA. Plates were incubated at 4°C for 72 hr, 25°C and 37°C for 24 hr.

### 5.3.4 Proteolytic activity

The proteolytic activity of all the isolates was carried out using skim agar that was prepared by adding 800 mL of distilled water to 23 g of nutrient agar (Code CM0003, Oxoid, UK), autoclaved and cooled to 45°C before adding 200 mL of skim milk (99.9% fat free, Devondale, Australia). Overnight cultures of the isolates were then spot-inoculated unto the plates and incubated at 25°C for 72 hr. Plates were observed for clear zone after 24, 48 and 72 hr. Zone of clearance was obtained and analysed as described by Alonso et al. (2017).

$$\text{Zone of clearance} = \frac{\text{Total diameter of zone of clearance - diameter of colony}}{\text{Diameter of colony}}$$

### 5.3.5 Lipolytic activity

The ability of the isolates to degrade lipids was investigated through the use of Tween 80 (Code P5188, Sigma, Germany). NA was prepared as above with 10 mL Tween 80 added instead of skim milk and autoclaved at 121°C for 15 minutes. The molten agar was poured into Petri dishes and allowed to solidify. Thereafter, 10 µL of overnight broth culture of the isolates was used to inoculate the plates while the plates were incubated as above and observed for clear zone.

### 5.3.6 DNase

DNase agar prepared according to manufacturer's instruction was used to investigate DNase production (Zhu et al., 2017). Methyl green (Code M6776, Sigma, Germany) was added to serve as indicator of zone of clearance.

### 5.3.7 Trimethylamine n-oxide (TMAO) reduction to trimethylamine (TMA)

The isolates were investigated for reduction of TMAO to TMA following the modified method of Dalgaard (1995b). The medium used consisted of peptone 2 g (Code LP0037, Oxoid, UK), Lab Lemco powder 0.3 g (Code L29, Oxoid, UK), yeast extract 0.3 g (Code LP0021, Oxoid, UK), ferric citrate 0.03 g (Code 28381, BDH, UK), sodium thiosulphate 0.03 g (Code S17, Ajax Chemicals, Australia), NaCl 0.4 g (Code 0314B92, Amresco, USA), $KH_2PO_4$ 0.4 g (BDH Chemicals, UK) $K_2HPO_4$ 0.575 g (Ajax Chemicals, Australia), $MgSO_4$ 0.05 g (Ajax Chemicals, Australia), L-cysteine 0.04 g (Code 37055, BDH, UK), 0.5 g TMAO - $2H_20$ ( Code 82H7709, Sigma, Germany), agar 0.4 g (Code LP0011, Oxoid, UK) and 0.1 g of tetrazolium chloride (Sigma, Germany) as redox indicator per 100 mL. L-cysteine and TMAO were filter-sterilised before adding to sterilised agar while 10 mL of prepared medium was poured into screw cap tubes. Each tube was inoculated with 20 µL of $10^3$ – $10^4$ CFU $mL^{-1}$ culture. The tubes were covered with sterile paraffin oil before incubating at 25°C. The tubes were observed daily for colour change due to reduction of TMAO and the production of $H_2S$ production from the precipitation of black FeS from both sodium thiosulphate and L-cysteine.

## 5.4 Molecular characterization

Ten $H_2S$-producing isolates were randomly selected from the 46 isolates that have been biochemically identified for molecular characterization Cultures were streaked on TSA and incubated overnight at 25°C. Thereafter, a loopful of each culture was then mixed with 100 µL of Prep Man buffer (Thermo Fisher Scientific) in 1.5 mL Eppendorf tube. The tubes were heated for 5 minutes at 100°C for cell lysis. Amplification of 750 bp region of the gene was carried out using universal primers (forward primer B27F - 5' - AGAGTTTGATCMTGGCTCAG - 3' and reverse primer: 1492R - 5' - GGYTACCTTGTTACGACTT - 3') and then sequenced using Applied Bio systems 3730 DNA analyser by Australian Genome Research Facility (AGRF, Melbourne Australia). The sequence chromatograms generated were edited using Bio-Edit software (Hall et al., 2011). The nucleotide sequences were then compared to published sequences via the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences of nearest neighbours identified using BLAST were then aligned with and phylogenetic analyses constructed using MEGA 7 software (Kumar et al., 2016). A Neighbour-joining tree was constructed using the maximum likelihood distances. The corrected sequences (Appendix A) were deposited in GenBank and were assigned accession numbers.

## 5.5 Spoilage potential

### 5.5.1 Preparation of inoculum

The isolates were inoculated into sterile TSA-yeast extract + 6% NaCl and incubated overnight. Thereafter, a sterile loop was used to touch the surfaces of the colonies and inoculated into freshly prepared 1 mL nutrient broth (NB) in cell culture plates before incubating at 25°C for 24 hr. Twenty microlitre was used to inoculate cooked mussel juice broth (MJB). Ten microlitre used to inoculate cooked mussel juice agar (MJA). Both MB and MJA were prepared as described in section 5.5.2 below.

### 5.5.2 Preparation of medium A - cooked mussel juice broth and medium B - cooked mussel juice agar (MJA)

Media A and B were prepared following modified methods of Parlapani et al. (2015) and Parlapani et al. (2017). Freshly harvested blue mussels (*Mytilus galloprovincialis*) was obtained as stated in section 5.2.1 and used in making mussel juice agar (MJA). Mussels were shucked with sterile knife while 100 g of mussel meat (gut excluded) was weighed and added to 400 mL of water purified by 0.2 µm filtration and reverse-osmosis (Pureau, Australia) purchased from Coles supermarket (Mowbray) and homogenised for 60 seconds. This was further divided into parts A and B with each part containing 50 g of mussel in 200 mL of chemical-free water. The homogenates were sieved to remove mussel flesh while 300 mL of chemical-free water was added to each part before autoclaving at 121°C for 15 minutes. The autoclaved mussel juice broth (MJB) was then allowed to cool at room temperature so as to allow the mussel meat debris to settle and then decanted into sterile Schott bottle.

Thereafter, 7.5 g of bacteriological agar was prepared with 100 mL of the chemical-free water and autoclaved as above. The prepared sterile agar was allowed to cool to 45°C before adding it to mussel juice part A while part B was used as mussel broth. Part A agar was then swirled for proper mixing and then poured into sterile Petri dishes.

### 5.5.3 Mussel protein degradation

MJA prepared above was used to investigate protein degradation potential among the isolates. It was prepared by adding 1.5% autoclaved agar to sterile MJB. After cooling to 45°C, the agar was poured into Petri dishes and used for protein degradation by spot-inoculating overnight cultures from TSA onto the dry surface of MJA. The plates were incubated at 25°C for 72 hr. The plates were observed for cleared zone (indicating protein degradation) which was measured after 24, 48 and 72 hr. Proteolytic activity was categorised into four levels, +, ++, +++, and ++++ corresponding to Diameter (D) of activity (mm) = 0; 1 mm ≤ D ≤ 6 mm; 7 mm ≤ D ≤ 11 mm and D ≥ 12 mm using mussel agar NPA (A) = No proteolytic activity; SPA (B) = Slight proteolytic activity; MPA (C) = Moderate proteolytic activity; STPA (D) = Strong proteolytic activity.

### 5.5.4 Production of off odour

Cooked mussel juice (MJB) was prepared as above. Thereafter, 0.4 g of $KH_2PO_4$ and 0.6 g of $K_2HPO_4$ were added to 100 mL of the cooked mussel juice and sterilised at 121°C for 15 minutes as described by Gram et al. (1987). Filter sterilised TMAO (1.6 g) and L-cysteine (0.4

g) were added while 10 mL of the prepared cooked mussel juice was distributed into screw capped tubes. Tubes were inoculated with isolates and incubated at 25°C for 10 days. Un-inoculated mussel juice samples were used as control.

## 5.6 Qualitative volatile metabolites analysis

### 5.6.1 Sample inoculation

Based on the results of the spoilage evaluation described above, isolate sw19 was selected and used for investigating volatile metabolites (VOC) production in the cooked mussel broth. The isolate was cultured overnight in peptone broth. This was then 5-fold serially diluted using sterile distilled water. Thereafter, 100 µL of the sample was added to 900 µL of sterile distilled water while 100 µL of inoculum as axenic culture was then used to inoculate 200 mL mussel juice broth. Un-inoculated mussel juice broth served as control. Both were incubated at 4°C for 10 days.

### 5.6.2 Headspace volatile metabolite (VOC) analysis using headspace solid phase micro extraction gas chromatography mass spectrometry (HS – SPME GC-MS)

The detection and identification of volatile metabolites produced by the axenic culture inoculated in the mussel juice broth was carried out using headspace solid phase micro extraction gas chromatography mass spectrometry (HS – SPME GC-MS) technology as described in Chapter 3 of this thesis. Briefly, the first step was the selection of suitable fiber capable of detecting broad range of volatile metabolites. Divinylbenzene –DVB /Carboxen –

CAR/ Polydimethylsiloxane – PDMS Stable flex, a 3 phases fiber was selected and then pre-conditioned using 10 mL of freshwater in screw capped vial for 20 minutes for decontamination purpose. The water sample was firstly incubated at 80°C for 30 minutes. The inserted fiber was then transferred into a GC - MS (Bruker 300 – MS TQ Mass Spectrometer) for less than 10 minutes. In the second step, 20 mL of mussel juice broth (both inoculated and control) in septum vial (40 mL) were incubated at 80°C for 30 minutes in an oven (Hewlett Packard 5590 series 11) to attain equilibrium of the volatile metabolites. Thereafter, the pre-conditioned fiber was inserted into the samples via the septum at room temperature for 20 minutes. Caution was taken not to allow the fiber to touch the sample before the exposed fiber was transferred into GC injector for 8-minute desorption of the volatile metabolites.

The GC MS used a split/splitless injector with full scan quadrupole detector equipped with an Agilent DB 5MS capillary column based on helium as carrier gas with flow rate of 1 mL $min^{-1}$ and an injector temperature of 270°C. Initially the holding temperature was 40°C for 4 min, then 80°C for 6°C/min and then ramping from 250°C to 290°C at 25°C $min^{-1}$. The mass detector used was ion mass/charge ratio (35-350 m/z) with full scan mode. Acquired chromatogram and spectral obtained was analysed using Mass Spectrometer (MS) Data review by marching each spectrum with available compounds in the National Institute of Standard and Technology (NIST, USA) database. A baseline of > 90 relative march was used as standard. Peak area of each spectrum was used to quantify volatile metabolites in the samples.

## 5.7 Statistical analysis

A paired t-test was used to evaluate significant differences at 95% confidence interval in the enzymatic activities of the isolates using IBM SPSS version 24 (USA). The peak area of each volatile metabolite was normalised to quantify the volatiles and data analysis was performed using GraphPad Prism 7 (GraphPad Software, CA 92037 USA).

## 5.8. Results

### 5.8.1 Isolation

There were no $H_2S$-producing bacteria isolated on day 0 (Table 5.1). Less than 20 colonies were observed on day 4 at 4 hr, 12 hr depuration and in the commercial packs (CM). On day 7, $H_2S$- producing bacteria were moderately present (colonies > 50) in samples from 4 hr depuration and commercial packs while colonies > 20 < 50 were observed in samples from 12 hr depuration. A similar pattern was observed on day 10 while the bacteria were abundant in all the samples on day 15. A total of 46 $H_2S$-producing bacteria were isolated and purified from pouch water (28 colonies) and mussel meat (18 colonies) (Figure 5.1).

**Table 5.1:** Occurrence and distribution of $H_2S$ - producing bacteria in depurated MAP live mussels.

| Sampling day | 4 hr | 8 hr | 12 hr | CM |
|---|---|---|---|---|
| **0** | - | - | - | - |
| **4** | + | - | + | + |
| **7** | +++ | + | ++ | +++ |
| **10** | +++ | + | ++ | +++ |
| **15** | ++++ | +++ | ++++ | ++++ |

- = no observed growth; + = barely present - few colonies < 20; ++ = slightly present - colonies > 20 < 50; +++ = moderately present - colonies > 50; ++++ = abundantly present – too numerous to count (TNTC).

**Figure 5.1**: Cultural morphology of $H_2S$-producing (black colonies) bacteria on Iron agar after 48 hr incubation at 25°C.

### 5.8.2 Biochemical and physiological characteristics

The results of the biochemical and physiological characteristics of the isolates were reported in Table 5.2. All the isolates could grow at 0 – 3% salt levels after 24 hr incubation. However, it was observed that after 24 hr's incubation, 65% (30/46) of the isolates could not grow at 6% salt concentrations. Plates were then further incubated for another 24 hr. It was observed that 50% of pouch water isolates and 61% of the isolates from mussel meat showed moderate proteolytic activity while 20% (9/46) of the isolates could not produce protease (Table 5.2). In this study, 35/46 of the isolates produced lipase. Isolates w9, w12, m17, w23 and w29 could not produce either enzyme. Only 2% of the isolates could not produce DNase while only 26% (12/46) of the isolates could reduce TMAO to TMA (Table 5.2).

**Table 5.2:** Biochemical characteristics of $H_2S$-producing bacteria isolated from MAP live mussels

| | Responses | |
|---|---|---|
| **Biochemical characteristics** | | |
| | **Positive (n=46)** | **Negative (n=46)** |
| Gram staining | - | 46 |
| Catalase | 46 | - |
| Oxidase | 46 | - |
| Salt tolerance (%) | | |
| 0 | 46 | - |
| 1.5 | 46 | - |
| 3 | 46 | - |
| 6 | 16/46 | 30/46 |
| Growth temperature ($^oC$) | | |
| 4 | 20/46 | 26/46 |
| 25 | 46 | - |
| 37 | 46 | - |
| $H_2S$ production | | |
| Kligler agar | 46 | - |
| Iron agar | 46 | - |
| TMAO reduction | 12/46 | 34/46 |
| Enzyme | | |
| Protease | 37/46 | 9/46 |
| DNase | 44/46 | 2/46 |
| Lipase | 35/46 | 11/46 |
| Carbon source utilization | | |
| D – sorbitol | - | 46 |
| L – arabinose | - | 46 |
| D – glucose | - | 46 |
| Sucrose | - | 46 |
| Amino acid utilization | | |
| Arginine | - | 46 |
| Lysine | - | 46 |
| Ornithine | - | 46 |

Adapted from Bergey's manual (Kersters and Vancanneyt, 2005);Beaz-Hidalgo et al. (2015); Dehaut et al. (2014) and Deng et al. (2014).

### 5.8.3 Molecular characterization

Ten isolates selected based on biochemical characteristics and TMAO reduction were sequenced. Sequences were compared with nucleotides sequences in the NCBI database and were deposited into GenBank with accession numbers MF599111 – MF599120 (Table 5.3). The closest relative of the isolates at 100% similarity was *S. baltica* SBO3, however, the phylogenetic tree constructed showed closeness to *S. baltica* 10735 which is a type strain (Figure 5.2).

**Table 5.3:** Molecular identification of selected isolates.

| Isolates | Genus* | Nearest neighbour | Similarity (%) | Accession numbers |
|---|---|---|---|---|
| **S1** | *Shewanella* | *S. baltica SB03* | 100% | MF599111 |
| **S2** | *Shewanella* | *S. baltica SB03* | 100% | MF599112 |
| **S3** | *Shewanella* | *S. baltica SB03* | 100% | MF599113 |
| **S4** | *Shewanella* | *S. baltica SB03* | 100% | MF599114 |
| **S5** | *Shewanella* | *S. baltica SB03* | 100% | MF599115 |
| **S6** | *Shewanella* | *S. baltica SB03* | 100% | MF599116 |
| **S7** | *Shewanella* | *S. baltica SB03* | 99% | MF599117 |
| **S8** | *Shewanella* | *S. baltica C422* | 99% | MF599118 |
| **S9** | *Shewanella* | *S. baltica SB03* | 99% | MF599119 |
| **S10** | *Shewanella* | *S. baltica SB03* | 100% | MF599120 |

*Phenotypic identification based on production of $H_2S$ and biochemical identification.

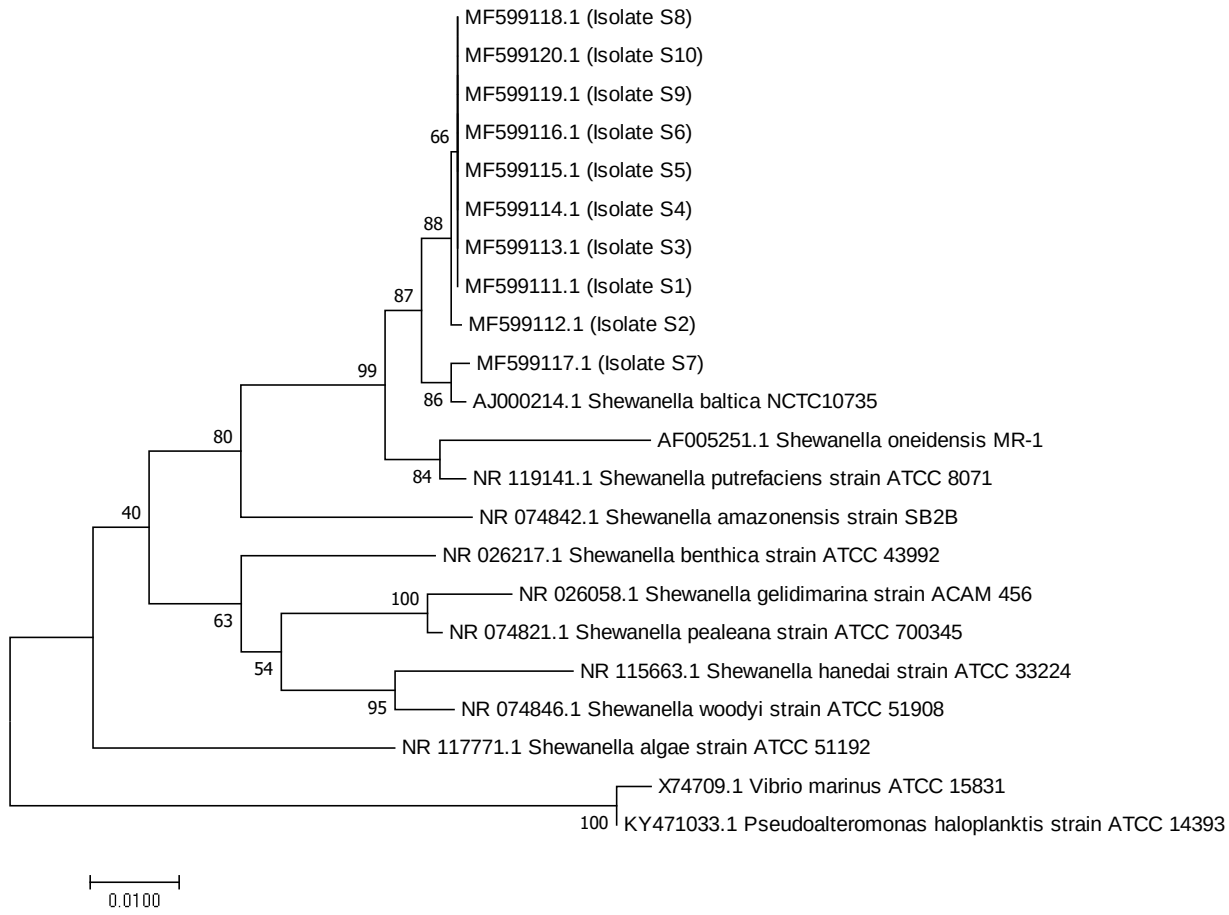


**Figure 5.2**: Phylogenetic affinities of $H_2S$ – producing isolates based on alignment of approximately 714 base pairs of the 16S rRNA gene. Neighbour-Joining tree using the Maximum Composite Likelihood distance; gaps and missing data were excluded from the analysis. The scale bar indicates of base substitutions per site. Evolutionary analyses were conducted in molecular evolutionary genetics analysis - MEGA7 (Kumar et al., 2016).

### 5.8.4 Spoilage potential of $H_2S$-producing bacteria

It was observed that the isolates could degrade mussel protein (Figure 5.3) and skim milk agar (Figure 5.4). The degradation of mussel protein and proteolytic activity on skim milk agar grouped the isolates into four: no proteolytic activity (NPA), slight proteolytic activity (SPA), moderate proteolytic activity (MPA), and strong proteolytic activity (STPA) (Figures 5.5).

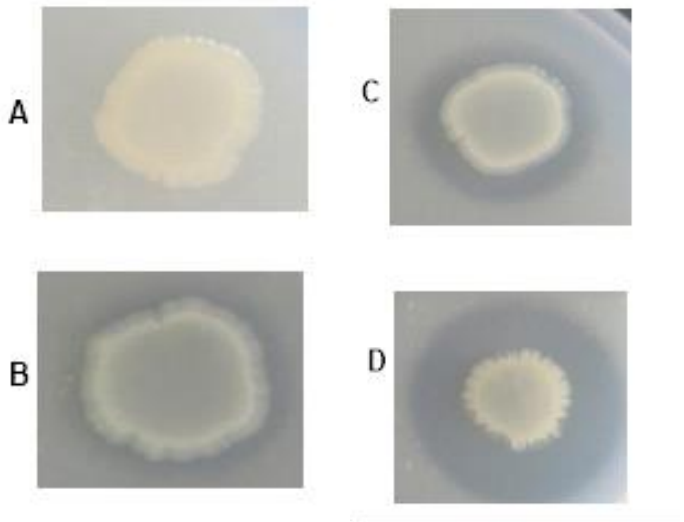


**Figure 5.3**: Representative proteolytic activity of isolates on mussel agar. Proteolytic activity was categorised into four levels, +, ++, +++, and ++++ corresponding to the diameter (D) of activity (mm) = 0; 1 mm ≤ D ≤ 6 mm; 7 mm ≤ D ≤ 11 mm and D ≥ 12 mm using mussel agar NPA (A) = No proteolytic activity; SPA (B) = Slight proteolytic activity; MPA (C) = Moderate proteolytic activity; STPA (D) = Strong proteolytic activity.

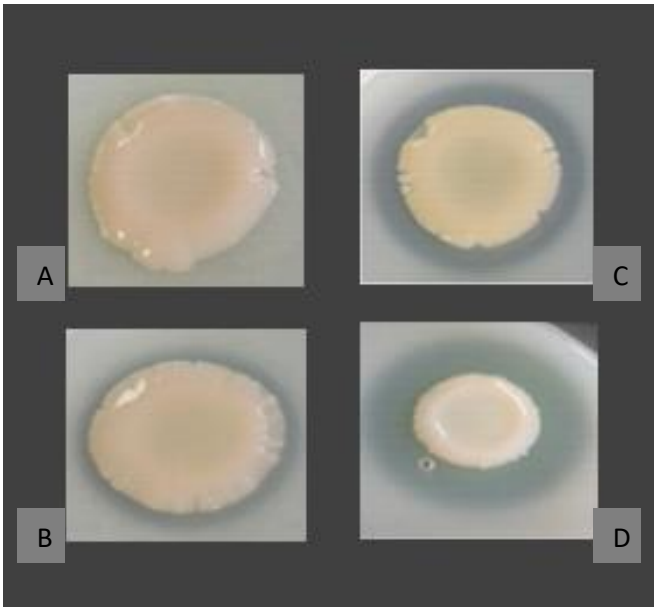


**Figure 5.4**: Representative proteolytic activity of isolates on skim milk agar. Proteolytic activity was categorised into four levels, +, ++, +++, and ++++ corresponding to Diameter (D) of activity (mm) = 1 mm ≤ D ≤ 2 mm; 3 mm ≤ D ≤ 7 mm and D ≥ 8 mm using skim milk agar. NPA (A) = o proteolytic activity; SPA (B) = slight proteolytic activity; MPA (C) = moderate proteolytic activity; STPA (D) = strong proteolytic activity.

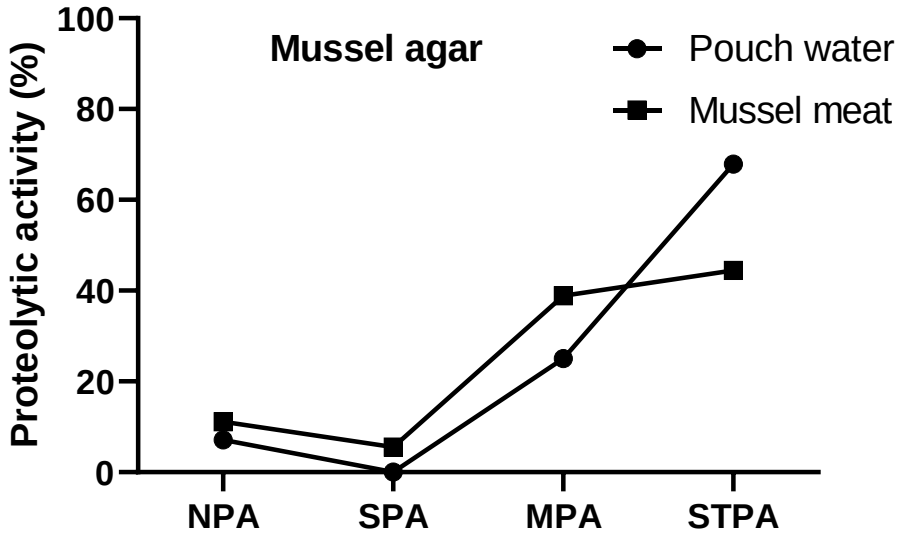


**Figure 5.5**: The summary of positive proteolytic activity (%) of isolates on mussel agar. NPA = No proteolytic activity; SPA = Slight proteolytic activity; MPA = Moderate proteolytic activity; STPA = Strong proteolytic activity**.**

### 5.8.5 Qualitative volatile metabolites analysis

A total of 27 volatile metabolites were obtained before inoculation and 44 volatile metabolites were obtained on day 10 after inoculation (Table 5.4).

**Table 5.4:** Total volatile metabolites detected by GCMS analysis (x = present) from cooked mussel juice broth (MJB) before inoculation with *Shewanella* isolate sw19 and after inoculation and incubated at 4°C for 10 days.

| **Volatile compounds** | **Before inoculation** | **After inoculation** |
|---|---|---|
| | **Day 0** | **Day 10** |
| *Aldehydes* | | |
| Hexanal | x | x |
| Heptenal | x | x |
| Benzaldehyde | | x |
| Nonanal | | x |
| Decanal | | x |
| Lilac aldehyde | x | x |
| C12 –C15 aldehyde | x | x |
| Tetradecanal | | x |
| Octadecanal | x | x |
| *Alcohols* | | |
| 3-Methylbutanol | | x |
| Heptanol | x | x |
| Octadienol | | x |
| Octenol | x | x |
| Nonedienol | x | x |
| *Ketones (5)* | | |
| Methyl heptanone | x | x |
| Octenone | | x |
| Nonanone | | x |
| Methyl phenyl oxime | x | x |
| *Nitrogen compounds (2)* | | |
| Indole | | x |
| *Sulphur compounds (4)* | | |
| Dimethyl sulphide | x | x |
| Dimethyl disulphide | x | x |
| Dimethyl trisulphide | | x |

**Table 5.4:** Total volatile metabolites detected by GCMS analysis (x = present) from cooked mussel juice broth (MJB) before inoculation with *Shewanella* isolate sw19 and after inoculation and incubated at 4°C for 10 days (Continued).

| Volatile compounds | Before | After |
|---|---|---|
| | Day 0 | Day 10 |
| ***Esters*** | | |
| Ethylbenzoic acid, butylester | | x |
| ***Acids*** | | |
| Fatty acid | x | x |
| | | |
| ***Furans*** | | |
| Pentylfuran 1 | x | x |
| Pentylfuran 2 | | x |
| | | |
| ***Volatile phenols*** | | |
| Methyl phenol | | x |
| Phenol | x | x |
| | | |
| ***Aromatic hydrocarbons*** | | |
| Toluene | x | x |
| 3,5-Octadiene | | x |
| Octadiene | | x |
| Ethylbenzene | x | x |
| 3-Ethylthiophene | | x |
| Xylene | | x |
| Thiohexene | | x |
| Naphthalene | x | x |
| Bis(dimethylethyl) benzene | | x |
| | | |
| ***Hydrocarbons*** | | |
| Tetramethyl pentane | | x |
| Undecane | | x |
| Dodecane | x | x |
| Tridecane | x | x |
| Pentadecane | | x |
| Heptadecane | x | x |

Some of the volatile metabolites such as hexanal, heptanal, lilac aldehyde, heptanol, C12-C15 aldehyde and octadecanal were found in cooked mussel broth before and after inoculation (Table 5.4). They all decreased during storage and could be contributing to the freshness flavour of mussels.

The following volatile metabolites were not obtained on day 0 but were obtained in inoculated samples after 10 days storage: nonanal, decanal, tetradecanal, 3 methylbutanol, octadienol, octenone, nonanone, dimethyl trisulphide, butylester, pentylfuran 2, methyl phenol, 3, 5 octadiene, octadiene, undecane, tetramethyl pentane, and pentadecane (Table 5.4).

## 5.9 Discussion

### 5.9.1 Isolation, physiological characteristics and molecular identification of $H_2S$-producing bacteria isolated from live mussels.

As stated in Chapter 4, effective depuration could help in reducing spoilage bacteria and extend the shelf-life of live mussels. The need to study the spoilage potential of either axenic or mixed cultures of spoilage bacteria such *Shewanella* and *Acidaminococcus* was suggested. This current therefore focuses on examining the spoilage potential of Shewanella due to production of $H_2S$ associated with the bacteria. The role of $H_2S$-producing bacteria such as *Shewanella* species in spoilage of fish and other seafood is well documented (Boskou and Debevere, 1998; Jorgensen and Huss, 1989; Lopez-Caballero et al., 2001; Zhu et al., 2017). However, there is no information on the role of *Shewanella* species on the spoilage of MAP live mussels.

The gradual increase in the number of $H_2S$-producing bacteria such as *Shewanella* species in seafood during storage has been described as contributor to spoilage (Gram and Dalgaard, 2002). Goulas et al. (2005) reported the isolation and identification of *Shewanella putrefaciens* in vacuum-packaged and MAP-shucked mussels (*M. galloprovincialis*) stored at 4°C. Bernardez and Pastoriza (2011) studied the quality of live mussels based on total viable count (TVC). However, Bernardez and Pastoriza (2013) reported high occurrence of *Shewanella* from the intervalvular liquid of live packaged mussels stored at 2°C and 7°C on the last day of storage.

The use of growth at 4°C and / or 37°C to differentiate *S. baltica* and *S. putrefaciens* has been a long-time practice (Fonnesbech et al., 2005). In this current study, about 55% (26/46) of isolates could not grow at 4°C after 72 hr incubation. However, all the isolates could grow at 25°C and 37°C (Table 2). This was different from the result obtained by Zhu et al. (2017) in which all *S. baltica* isolated from spoiled shrimp were not able to grow at 37°C. Ge et al. (2017) reported that 8 isolates of *S. baltica* strains obtained from spoiled large yellow croaker could not grow at 37°C but could grow at 4°C, 25°C and 30°C. Similarly, Dehaut et al. (2014) reported the aerobic growth of *S. baltica* isolated from spoiled whiting from 4°C to 30°C but not at 37°C. The result of this current study was contrary to that of Ziemke et al. (1998) who isolated *S. baltica* strains from Baltic sea that were unable to grow at 37°C. However, the results were similar to that of Fonnesbech et al. (2005) in which some strains of *S. baltica* isolated from fish were able to grow at 37°C. No reported study on isolation of *S. baltica* from MAP live mussels could be identified in the literature so as to compare with the results of this current study. Serio et al. (2014) observed that *S. baltica* could grow at 37°C after 3 / 6 days incubation while isolates were incubated for 24 hr - 72 hr (1 – 3 days) in this present study.

The results of this current study indicated that 45% of the isolates were psychrotrophic. *S. baltica* isolated from different seafood showed variable psychrotrophic abilities (Dehaut et al., 2014; Serio et al., 2014). This highlights the spoilage potential of *S. baltica* at refrigeration temperatures. Variation in growth at different temperatures could be as result of the different techniques used to assess growth (Tryfinopoulou et al., 2007). Some of the studies used broth for growth studies with or without salt (NaCl) added while some used agar with

or without yeast extract supplement. The type of medium used will determine available sources of nutrients for the bacteria and hence could result in false positive or false negative results. Media used were not always stated thereby making repetition of some of the studies difficult. Tryfinopoulou et al. (2007) stated that seafood storage temperature could determine the type of *Shewanella* species that will be prevalent in the samples. The ability of 20 out of 46 isolates to grow at 4°C in this current study, signifies the potential of *Shewanella* to cause spoilage of MAP live mussels if stored at low temperature.

The results of this study also demonstrated the ability of the isolates to grow and survive in a salt water environment. Salt tolerance has also been used to differentiate *Shewanella* species (Fonnesbech et al., 1997). All isolates in this current study were observed to grow on agar supplemented with 6% NaCl after 72 hr incubation. This result was similar to that of Serio et al. (2014). They observed that all *S. baltica* tested were able to grow at 6% salt level but observed variation in the growth at increased salt concentrations (8 – 12%). (Fonnesbech et al., 2005) also observed similar variation among 80 strains of *S. baltica* isolated from ice-stored fish. In contrast, Ge et al. (2017) reported that only one of the 10 strains of *S. baltica* isolated from fish (yellow croaker) stored at 4°C could grow at 6% salt concentration.

Ge et al. (2017) used an assay kit for bacterial protease and photometric methods to assess protease activity and reported that 6 of 8 (75%) isolates of *S. baltica* produced protease. Kulakova et al. (1999) also reported that *Shewanella* sp. produced protease at cold storage temperature using colorimetric method. Serio et al. (2014) stated that proteolytic activity in

*Shewanella* is strain dependent. Similar to the results of this current study, Fonnesbech et al. (2005) observed production of DNase in *S. baltica* isolated from ice-stored fish.

All the isolates in this current study were biochemically identified as *Shewanella* species. However, there was need to use molecular method to identify the isolates at species level as biochemical identification is not sufficient to distinguish species of *Shewanella* (Dehaut et al., 2014). For example, in this current study, salt and temperature tolerance were observed not to be reliable to classify *Shewanella* species. The closest relative of the isolates obtained in this current study at 100% similarity was *S. baltica* SBO3 isolated from spoiled large yellow croaker (*Pseudosciaena crocea*) stored at 4°C (Ge et al., 2017). Ge et al. (2017) reported that *S. baltica* strains from the spoiled yellow croaker produced black pigment except for 2 strains that produced white colonies on Iron agar. All the isolates could reduce TMAO, grow between 4°C to 30°C, produced protease and lipase enzymes. In another study, Dehaut et al. (2014) isolated *S. baltica* from spoiled whiting fish stored at 8°C for 6 days. Both 16S rRNA and gyrB genes were used for molecular characterization of *S. baltica* in their study. They reported *S. baltica* caused spoilage of the fish. Serio et al. (2014) reported occurrence of *S. baltica* in refrigerated swordfish and red tuna indicating S. baltica can cause spoilage of a wide range of seafood. In a recent study, Khazandi et al. (2017) isolated *S. baltica* from salmon and whiting fillets stored at 4°C. Results from this current study and previous studies implied that *Shewanella* species are part of normal microbiota of farmed or wild seafood and can become prevalent at cold storage temperatures.

No studies have reported *S. baltica* from live mussels. The results of this study indicated that *S. baltica* is capable of degrading protein and hence could degrade mussels in the packs. Evidence of this was the sulphide odour smelt in the packs as they were opened during storage. As a result of this observation, the headspace volatile metabolites produced by pure cultures of *S. baltica* were investigated in cooked mussel broth.

### 5.9.2 Spoilage potential and volatile metabolite production of *S. baltica* isolated from live mussels

There have been various reports on the use of TMAO reduction as seafood spoilage index. However, no such study has been carried out among isolates of *Shewanella* obtained from mussels. Serio et al. (2014) observed that 3 out of 16 strains of *S. baltica* from fish could not produce TMA. In this study, 12 out of 46 isolates could reduce TMAO and 9 of the isolates were from the pouch water samples. Dehaut et al. (2014) also reported that 4 strains of *S. baltica* isolated from spoiled ice stored whiting fish could reduce TMAO at 25°C. As only 61% of the isolates produced sulphide off-odours, reduction of TMAO would appear to be isolate-dependent rather than generalised behaviour among *Shewanella* species.

Volatile metabolites grouped into alcohols, aldehydes, phenols, furans, ketones, esters, organic acids, aromatic hydrocarbons, alkanes and nitrogen - and sulphur -containing compounds have been used to indicate freshness or spoilage of seafood. (Raina et al., 2010) reported that aldehyde, ketone and alcohol volatile metabolites in seafood originated from enzymatic activities of lipoxygenase on unsaturated fatty acid in seafood. In this study, some volatile metabolites were consistently present while others varied over time as reported in

Chapter 3 of this thesis. This was observed by Parlapani et al. (2017) in their recent study on microbial volatile metabolites obtained from inoculated seabream fish broth stored at 0 – 2°C. In this current study, benzaldehyde, nonanal, decanal and tetradecanal could be attributed to metabolic products of *S. baltica* strain MSSA1 because they were detected on day 10 in inoculated cooked mussel broth, but not in the control. This contrasted with the results of Parlapani et al. (2017) who reported the presence of the aldehydes, nonanal and decanal in fish broth on day 0. Hexanal, heptenal, lilac aldehyde, octadecanal and C12-C15 aldehyde were present in high amount in mussel broth before inoculation, but they decreased by day 10 after inoculation.

Alcohols, octenol, nonedienol and heptanol were detected on day 0 before inoculation and therefore could not be attributable to *Shewanella*. Octadienol and 3 methyl 1-butanol were only detected on day 10 post inoculation therefore were produced from microbial activity of the *Shewanella* isolate examined. Parlapani et al. (2017) also reported production of 3 methyl butanol from the activity of *Shewanella* in fish stored in air and at gas atmosphere of $CO_2$: 60%, $O_2$: 10%, $N_2$: 30%. Methyl heptanone was detected on day 0 while octenone and nonanone were detected on day 10 of the mussel broth incubation, suggesting that octenone and nonanone were from the activity of *S. baltica.* Indole was the only nitrogen compound detected on day 10 after inoculation. Hence it could be attributed to microbial activity because sterile mussel broth and pure culture of *Shewenella* strains were used. This was like the result of Snellings et al. (2003) who reported indole as the product of microbial activity in shrimp.

Dimethyl sulphide (0.6%) and dimethyl disulphide (0.4%) were detected in low concentrations on day 0 but became far more concentrated by day 10 (5% and 34% respectively). Studies have shown that dimethyl sulphide could be derived from the breakdown of dimethylsulphoniopropionate (DMSP) commonly found in marine algae (Tuckey et al., 2013), which may explain its presence prior to the inoculation of the MJA. It could also be produced as by-product of gut microbiota of seafood or enzymatic activity (Kiene, 1990; Sarnoski et al., 2010). Mussels used in this study were harvested from a mussel farm where the mussels feed mainly on naturally-occurring marine algae. The observed increase in dimethyl sulphide and dimethyl disulphide is similar to a report of Parlapani et al. (2017). This noticeable increase in this current study could only be attributed to the activity of *S. baltica* because no algae were present in the medium. The genus *Shewanella* has been mentioned among marine bacteria taxa that can degrade DMSP (Raina et al., 2010). However, sterile mussel broth was used in this study, therefore, DMS may not have resulted from enzymatic degradation of DMSP. Dimethyl disulphide was more and significantly increased than the other two sulphur compounds. This could be from the degradation of protein or amino acid in the mussel broth. Guen et al. (2000) reported detection of dimethyl disulphide in wild live mussels but did not report the source of the volatile metabolite. Dimethyl trisulphide could be attributed to metabolic activity of *S. baltica*. Zhang et al. (2010) reported dimethyl trisulphide in deteriorated live clam (*Sinono-vacula constricta*). Unlike the result of Zhang et al. (2010) who found that trimethylamine was the prevailing volatile metabolite in mussels, dimethyl disulphide was more abundant in deteriorated mussel broth in this current study, constituting 34% of VOC.

Butyl ester was identified only on day 10. This implies that the metabolite must have originated from microbial metabolism. Only one fatty acid (stearic acid) was identified on day 0 and day 10 in inoculated and un-inoculated samples. Mussels have been reported to be rich in fatty acids such as monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and amino acids (Bongiorno et al., 2015; Noseda et al., 2012; Varlet and Fernandez, 2010) mostly obtained from their diet (Li et al., 2013). Fernandez-Reiriz et al. (2015) reported that fatty acids are present in different organs of both male and female blue mussels (*M. galloprovincialis*). These nutritional components of mussels could serve as precursors for either freshness or spoilage volatile metabolites due to microbial, enzymatic activities and autolytic degradation. Pentylfuran 1 and Pentylfuran 2 were both identified in this study. However, only pentylfuran 2 was identified on day 10 as spoilage VOC. It was not detected in the samples on day 0 before inoculation. Other furans such as 2-propyfuran and 2, 3-dihydrofuran are found in Greenshell mussels (Tuckey et al., 2013). Other volatile metabolites identified are methyl phenol and phenol. In this study, phenol was detected on day 0 and 10 while methyl phenol was identified only on day 10. Phenol characterised with objectionable smell was used as indicator of seafood spoilage (Gram, 2009). All the VOC identified as either freshness or spoilage VOC in this Chapter were also observed in Chapter 3. For example, hexanal that was identified as a freshness VOC on day 0 in Chapter 3 was observed in the mussel meat of depurated mussels in Chapter 5. Similarly, indole identified as spoilage VOC in the mussel meat of undepurated and commercial packs in Chapter 3 was also obtained on day 10 in this study (Chapter 5).

## 5.10 Conclusion

Under the current commercial practice, live mussels only have a maximum of 10 days shelf-life. Understanding spoilage mechanisms and attendant spoilage indices will help in designing effective mussel quality protocols and shelf-life extension. The results of this study showed that live mussels harbour $H_2S$-producing bacteria, one of which was identified as *S. baltica* and was part of the normal flora. The *S. baltica* isolates detected were psychotrophic and halotolerant. Biochemical methods could not differentiate isolates at species level, however, 16S rDNA sequencing indicated that the isolates fall within a cluster of *Shewanella* sequences containing both *S. baltica* and *S. putrefasciens*. Analysis of VOCs produced by cooked mussel broth indicated that heptanal, octanal, hexanal and lilac aldehyde are freshness indicators, while dimethyl trisulphide, nonanal, decanal, phenol, pentyl furan 2 and octadienol were associated with spoilage by *Shewanella* after 10 days at 4 degrees. Theses VOCs were likely produced by *Shewanella*. Monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and amino acids present in mussels could serve as precursors for either freshness or spoilage volatile metabolites due to microbial activities and or autolytic degradation. Volatile metabolites could therefore be used to assess freshness and spoilage of MAP mussels using HS-SPME GC-MS. The method is non-destructive and can be used to monitor the quality of live mussels, however, it is expensive. Therefore, there is need to develop easy to use, inexpensive and simple tool that can be used to monitor the quality of live mussels during storage.

# Chapter 6: Development of a draft quality index method tool for evaluation of sensory quality and shelf-life of modified atmosphere packaged live mussels

## 6.1 Introduction

Consumers rely on visual, olfactory and organoleptic evaluations to determine freshness and palatability of seafood (Orban et al., 2002). The freshness of seafood reduces with storage time or days due to spoilage that could be caused by autolytic, microbial or enzymatic activities (Ocaño-Higuera et al., 2011). Observable changes in seafood during such as spoilage could be detected by human senses (Barbosa and Vaz-Pires, 2004). However, reliable methods for freshness evaluation are needed.

According to Olafsdóttir et al. (1997), sensory evaluation involves use of human senses of smell, sight, taste, hearing and touch to evaluate food and analytical interpretation. One of the sensory evaluation methods developed for seafood industry is the Quality Index Method (QIM), a reliable method of freshness assessment based on visual and olfactory examination of the quality of seafood (Billar dos Santos et al., 2014). QIM was developed for assessment of fish freshness at the Tasmania Food Research Unit (TFRU), Commonwealth Scientific and Industrial Research Organization, Australia by Bremner (1985). It is a descriptive, simple and fast method for freshness assessment (Bonilla et al., 2007). It involves objective evaluation and the use of observable sensory or quality attributes and parameters with a demerit scoring system that ranges from 0 to 3 (Bonilla et al., 2007; Bremner, 1985). The sum of the scores (demerit) for each quality attributes and parameter is known as the Quality Index – QI (Barbosa and Vaz-Pires, 2004).

QIM has been used to evaluate freshness quality and predict shelf-life different seafood especially fish. For example, Alasalvar et al. (2001) observed that the freshness of ice-stored seabream correlated with QIM and predicted 17-18 days as its shelf-life. Billar dos Santos et al. (2014) reported that the QIM developed for Acoupa weakfish (*Cynoscion acoupa*) correlated with storage time and concluded it was useful for freshness evaluation. Lanzarin et al. (2016) also developed QIM with 18 demerit limits and predicted the shelf-life of gutted Amazonian Pintado stored for 30 days at 0°C± 0.5°C (*Pseudoplatystoma fasciatum* × *Leiarius marmoratus*) to be 12 days, which correlated with microbial count and physicochemical attributes. The shelf-life of hybrid gutted ice-stored hybrid tambatinga (*Colossoma macropomum* × *Piaractus brachypomum*) was estimated to be 10 days (Ritter et al., 2016). Goncalves et al. (2015) developed QIM for spiny lobster (*Panulirus argus*) stored on ice and concluded that the tool can be used by consumers to evaluate the freshness of lobster. QIM has also been developed for both cooked black tiger (*Penaeus monodon*) and king prawns (*Melicertus plebejus*) (Boulter et al., 2006). However, they did not state the shelf-lives of the prawns in their study.

Despite the development of QIM for the evaluation of the freshness of different seafood, no QIM has been developed for the assessment of freshness and shelf-life prediction of modified atmosphere packaged (MAP) live mussels. Therefore, this study aimed to develop a Quality Index Method (QIM) tool for the evaluation of sensory quality and prediction of the shelf-life of live mussels. The QIM was then compared with mussel quality parameters such as total viable count (TVC), total ammonia nitrogen (TAN) and headspace oxygen.

## 6.2 Materials and methods

### 6.2.1 Sampling, packaging and storage of MAP live mussels

Freshly harvested standard sized (average length 80.33 ± 7.50 mm and width 47 ± 2.28 mm) mussels were obtained from commercial farm from east coast Tasmania, Australia. Samples were transported in iced-Styrofoam boxes to the laboratory. Samples were immediately depurated for 8 hr at 4°C before 6 – 8 mussels were packaged with the gas composition of 80% $O_2$ and 20% $N_2$. Prior to packing, 50 mL of sterile freshwater was added to the pouch to provide a humid environment for the mussels and to mimic commercial practice. Commercially packaged (thereafter referred to as Commercial packs - CP) samples depurated for 1-2 hr prior to packing with 75 – 80% headspace oxygen were used as reference. All samples were stored at 4°C for 15 days. Storage at 4°C (refrigeration) was used instead of 0°C (ice storage) due to commercial practise, consumers preference for live mussels and reduction stress that can lead to early mortality if stored at 0°C. Combining MAP with cool storage seems more practical for extending shelf-life than ice storage.

### 6.2.2 Development of Quality Index Method (QIM) tool

The development of QIM followed modified methods of Goncalves et al. (2015) and Billar dos Santos et al. (2014). Ethical approval (H0016651) from the human research ethics committee (HREC), University of Tasmania was firstly obtained (Appendix B). Voluntary assessors were publicly recruited. Two training sessions were held for assessors to understand the QIM tool and its scoring. Four assessors (3 males and 1 female) were recruited. However, due to the unavailability of one of the male assessors during one of the

sessions, only 3 assessors completed the training. MAP live mussels stored at 4°C were presented to the assessors at different storage times (0, 4, 7, 10 and 15 days). On each sampling day, a total of nine packs were presented to the three assessors. Each assessor was presented with triplicate packs to score. However, the initial headspace oxygen was firstly measured before presenting the packs to the assessors. The levels of headspace oxygen and $CO_2$ were analysed using a benchtop gas - analysing machine (PBI Dansensor Checkmate II Headspace Analyzer). A rubber septum was firstly placed on the pack before inserting a sucking needle after initial calibration with ambient air (Milne and Powell, 2014). The obtained gas levels were reported as percentages (%).

Upon presenting the samples to the assessors in triplicate, each sample was observed for any organoleptic change in quality attributes and parameters. The assessors were independent of each other. Each quality parameter and attribute were assigned demerit points from 0 – 3. As storage time of increases, the demerit score also increases. The total demerit points obtained by each assessor was then plotted against storage time to give a linear relationship that can then be used to predict the shelf-life of live mussels (Bernardi et al., 2013; Cyprian et al., 2014; Cyprian et al., 2008). Other quality parameters observed were further described below.

### 6.2.3 Mortality

Upon opening, any mussel found to be gaped and not able to close after gentle tapping was recorded as dead. Mortality was recorded in percentage (%) of dead mussels out of the total in the packs (Bernardez and Pastoriza, 2013).

### 6.2.4 Microbiological analysis

Each sample was microbiologically analysed immediately after sensory evaluation (Billar dos Santos et al., 2014; Goncalves et al., 2015). The total viable count (TVC) was investigated using a spread plate method on Standard Plate Count Agar (LP0011, Oxoid, England). Plates were incubated aerobically for 48 hr at 25°C. Colonies on each plate were counted as colony-forming units (CFU) and log transformed to log CFU $g^{-1}$ or log CFU $mL^{-1}$. Treatments were assessed in triplicate.

### 6.2.5 Physicochemical analysis

At each sampling point, the pH of homogenised raw mussel meat and of pouch water was determined using a pH meter (ATI Orion Research Model 250A, USA) at 25°C (Silva et al., 2010). The total ammonia nitrogen ( TAN mg $mL^{-1}$) of the pouch water was determined using a commercial ammonia testing kit (API, USA) following manufacturer's instructions.

### 6.2.6 Meat condition index (MIC)

Five selected raw mussels were weighed (wet weight) before cooking for 3 minutes in a microwave (Palsonic, China) at 240 volts and 1250 watts. Thereafter, the mussel meat and shell were weighed together, and mussel meat weighed separately (Çelik et al., 2012; Okumuş and Stirling, 1998).

### 6.2.7 Statistical analysis

The mean of TVC, pH, mortality, headspace gas and MCI were plotted individually against sampling period using GraphPad Prism version 7 (GraphPad Software, CA 92037 USA). Analysis of variance (ANOVA) was used to compare means of each parameter based on Tukey's test and a significant p value of 0.05 was used. Linear regression was used to obtain the quality index model and multivariate analysis (PCA) was used to determine which of the measured parameters could most impact freshness of the live mussels. XLSTAT software for windows version 2017 (Adin soft, Paris, France) and SPSS were used for statistical analyses.

## 6.3 Results

### 6.3.1 Development of Quality Index Method (QIM)

A total of 3 quality attributes, 7 parameters with 28 descriptors and 21 demerit points were identified and described for the development of a QIM that can be used for live mussels (Tables 6.1 and 6.2). These attributes, parameters and descriptors were then used for validation of the tool. Live mussels were assigned with two parameters: shell appearance and gaping; raw mussel meat was assessed based on three parameters: odour, texture and appearance, while pouch water was assessed based on odour and appearance.

The demerit points of the quality parameters increased with storage days (Figure 6.1). Among the seven quality parameters, only five parameters (appearance of raw meat, texture and odour of raw meat, appearance and odour of pouch water) contributed most to the demerit points and therefore were used to define the shelf-life of live mussels. There was no significant difference ($p>0.05$) in these parameters from day 0 to day 7, but they were significantly different ($p<0.05$) between day 10 and 15. An objectionable smell and mortality occurred on day 10.

Since the quality parameters can be easily used to obtain the QI, the exact storage days can then be estimated, and shelf-life predicted with the developed the exact storage days can then be estimated with the linear model obtained. QI = 1.261 x storage days - 1.6789 ($R^2$ = 0.9546, n = 3) (Figure 6.2). As storage day increases, the QI increases. At QI = 0, the mussels were fresh (storage days = 0) while at QI = 19, (storage days = 15) the mussels were

completely spoilt (complete loss of freshness). At the time of rejection (day 10), the QI was 11. The rejection of cooked meat had occurred sometime after day 10 and on day 15 (Figure 6.4). The model was used to group the freshness of live musses to be very fresh (QI = 0 – 4); fresh (QI = 5 – 8), slightly fresh (QI = 9 – 12); shelf-life ended (QI = 13 – 19).

**Table 6.1:** Quality Index Method (QIM) tool developed for modified atmosphere packaged live mussels stored at 4°C

| Quality attributes | Parameters | Descriptions | Demerit points |
|---|---|---|---|
| Live mussel | Shell appearance | Clean | 0 |
| | | Dirty | 1 |
| | | Clean broken | 2 |
| | | Dirty broken | 3 |
| | Mussel gaping | Closed | 0 |
| | | Slightly gaping - closes on tapping | 1 |
| | | Moderate gaping closes slowly | 2 |
| | | Wide gaping - remained opened on tapping | 3 |
| Raw mussel meat | Odour | Fresh sea smell | 0 |
| | | Faint sea smell | 1 |
| | | Slightly fishy – would just eat | 2 |
| | | Very fishy, putrid - would not eat | 3 |
| | Texture | Firm | 0 |
| | | Slightly firm | 1 |
| | | Soft | 2 |
| | | Very soft | 3 |
| | Appearance | Bright mantle, bright gills, white adductor muscle | 0 |
| | | Dull mantle, pale gill, white adductor muscle | 1 |
| | | Soft adductor muscle, disoriented gill | 2 |
| | | Soft mantle, soft disoriented gill, dull adductor muscle | 3 |
| Pouch water | Odour | Fresh sea smell | 0 |
| | | Faint sea smell | 1 |
| | | Slightly fishy – would just eat | 2 |
| | | Very fishy, putrid - would not eat | 3 |
| | Appearance | Clear | 0 |
| | | Slightly greenish/sea | 1 |
| | | Brownish / obvious turbidity | 2 |
| | | Brown / Heavy turbidity | 3 |
| **Total demerit** | | | **0-21** |

**Table 6.2:** Description of sensory qualities / attributes of live blue mussels

| Parameters | Descriptions of sensory qualities / attributes of modified atmosphere packaged live mussels |
|---|---|
| **Live mussel**<br><br>Shell appearance | **Clean** - glossy (glistening with moisture) blue - black shell devoid of seaweed, debris, barnacles, byssal threads and sediment<br>**Dirty** - presence of barnacles, byssal threads, seaweed and sediment<br>**Clean broken** - clean but cracked shell, crushed shell exposing meat, dislocated shell, hole in shell<br>**Dirty broken** - dirty with cracked shell, crushed shell, dislocated shell, hole in shell |
| Mussel gaping | **Closed** - no gaping (retains intravalvular fluid), both valves closed firmly without any visible space in between them.<br>**Slightly gaping** (1-2mm) - bivalve shell opens apart but close on gentle tapping within 10 – 20 seconds. Gills and or mantle not exposed.<br>**Moderate gaping** (3-5mm) – bivalve shell open but closes slowly on gentle tapping > 20 seconds. Gills and mantle exposed<br>**Wide gaping** (> 5mm) – bivalve shell remained open on gentle tapping due to weak adductor muscle. Gills and mantle are exposed. |
| **Raw mussel meat**<br><br>Odour | **Fresh sea smell -** characteristic of sea smell<br>**Faint sea smell -** faint characteristic of sea smell<br>**Slightly fishy -** would just eat<br>**Very fishy, putrid -** sulphide/ammoniac smell - would not eat |
| Texture | **Firm -** elastic when rubbed between fingers<br>**Slightly firm** - less elastic when rubbed between fingers<br>**Soft -** dissolving mantle when rubbed between fingers.<br>**Very soft -** dissolved mantle when rubbed between fingers |

**Table 6.2:** Description of sensory qualities / attributes of live blue mussels (Continued)

| Parameters | Descriptions of sensory qualities / attributes of modified atmosphere packaged live mussels |
|---|---|
| Appearance | **Bright mantle, bright gills, white adductor muscle –** bright creamy mantle (male), orange mantle (female), shiny brown gills<br>**Dull mantle, pale gill, white adductor muscle -** dull creamy mantle (male), orange mantle (female), pale brown gills<br>**Soft adductor muscle, disoriented gill -** dissolving mantle, gills not intact, adductor muscle easily pulled apart separating the valves<br>**Soft mantle, soft disoriented gill, dull adductor muscle -** dissolved mantle, dissolved gill, brown intravalvular fluid |
| **Pouch water** | |
| Odour | **Fresh sea smell -** characteristic of sea smell<br>**Faint sea smell -** faint characteristic of sea smell<br>**Slightly fishy -** would just eat<br>**Very fishy, putrid -** sulphide/ammoniac smell - would not eat |
| Appearance | **Clear -** clear without particles / debris / sediment / pseudo faeces<br>**Slightly turbid -** debris / sediment / pseudo faeces<br>**Turbid/brownish -** turbid due to debris / sediment / pseudo faeces and onset spawning<br>**Very turbid and brown -** very turbid mostly due to spawning and or dissolved tissue |

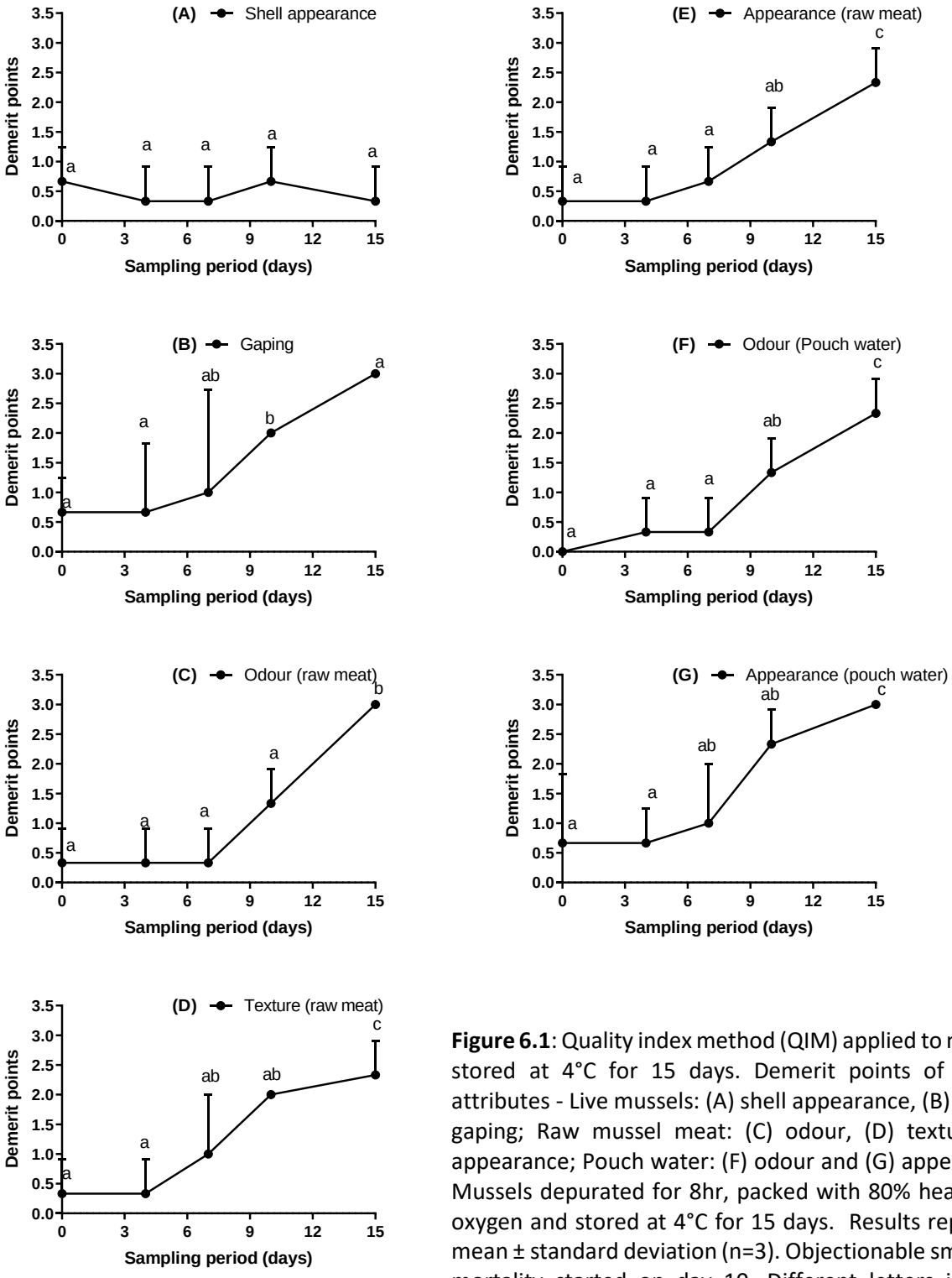


**Figure 6.1**: Quality index method (QIM) applied to mussels stored at 4°C for 15 days. Demerit points of quality attributes - Live mussels: (A) shell appearance, (B) mussel gaping; Raw mussel meat: (C) odour, (D) texture, (E) appearance; Pouch water: (F) odour and (G) appearance. Mussels depurated for 8hr, packed with 80% headspace oxygen and stored at 4°C for 15 days. Results represent mean ± standard deviation (n=3). Objectionable smell and mortality started on day 10. Different letters indicate significant difference at $p \leq 0.05$ within each characteristic based on Tukey's multiple comparisons test using IBM SPSS statistical software (version 24).

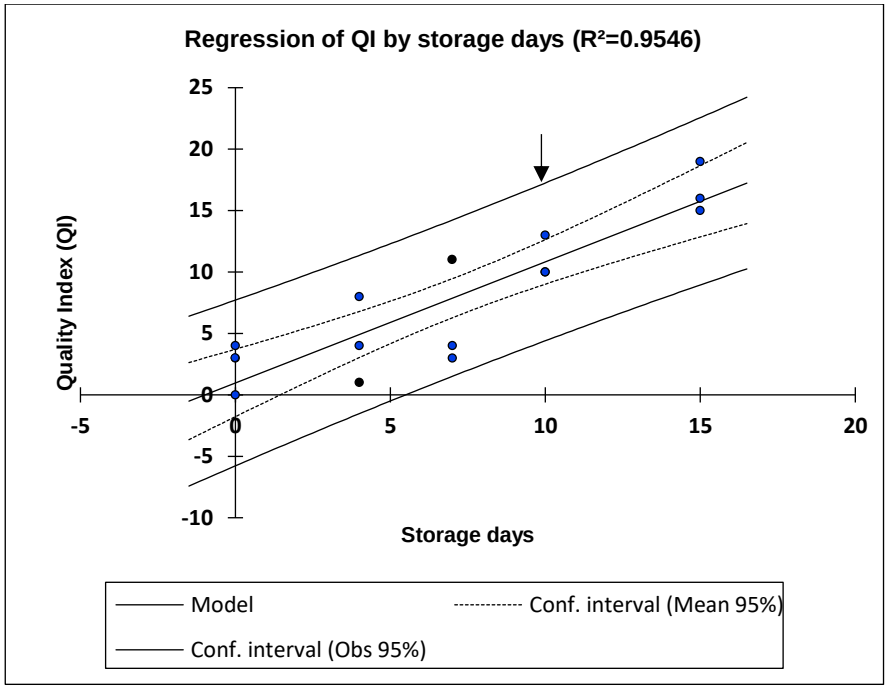


**Figure 6.2:** Linear regression of the storage days and demerits (quality index). The high $R^2$ indicates that storage days explained 95.5% of the variation of the Qi score. The directional arrow indicates rejection day by the assessors.

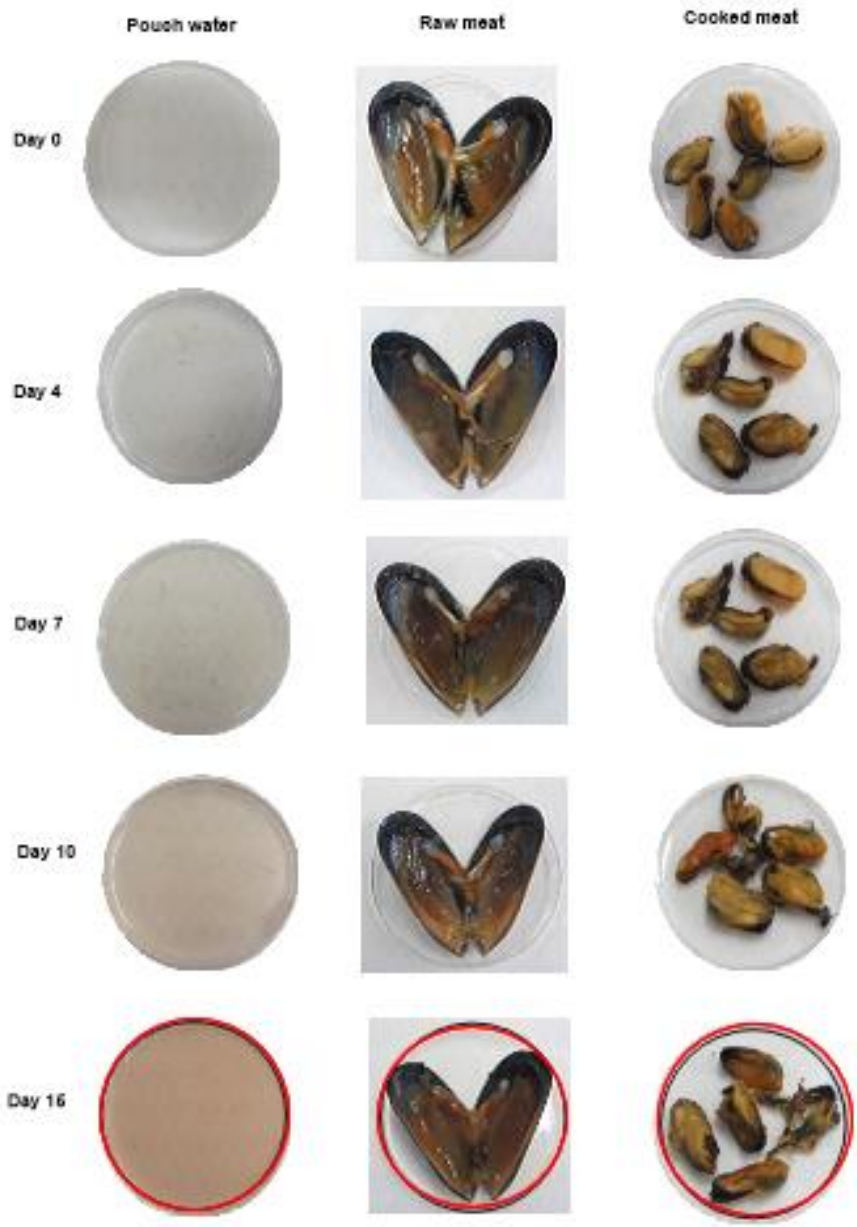


**Figure 6.3:** Representative pouch water, raw mussel meat and cooked meat of Lab-packaged samples stored at 4°C for 15 days. Red circles on day 10 indicate onset of objectionable sulphide smell in pouch water while red circles on day 15 indicate objectionable smell in the pouch water, and raw mussel meat (degraded), and palatability rejection of cooked meat.

### 6.3.2 Headspace oxygen and $CO_2$

There was no significant difference ($p>0.05$) in the headspace oxygen of the Lab-packaged samples and the Commercial packs (CP) on day 0 (Figure 6.4A). The headspace oxygen in the treatment did not significantly decline over days 0, 4, 7 and 10, but did decrease significantly to a mean of 58% on day 15. During this time, it remained significantly higher than the headspace oxygen in the Commercial packs (CP) (apart from day 7). In the Commercial packs (CP), the biggest changes occurred between day 0 and 4 (to a mean of 57.30%) and between day 10 and 15 (to a mean of 8.09%).

A steady increase in $CO_2$ was observed in both treatment and Commercial packs (CP) with measurable $CO_2$ appearing first in the Commercial packs (CP) on day 4 (Figure 6.4B). There was no significant difference ($p>0.05$) in the $CO_2$ detected in the treatment versus the Commercial packs (CP) on days 7, 10 and 15. Over time, $CO_2$ in the treatment and in the Commercial packs (CP) was 7.50 – 9.43% (day 7), 10.3 – 12.12% (day 10) and 18.27 – 23.60% (day 15) respectively.

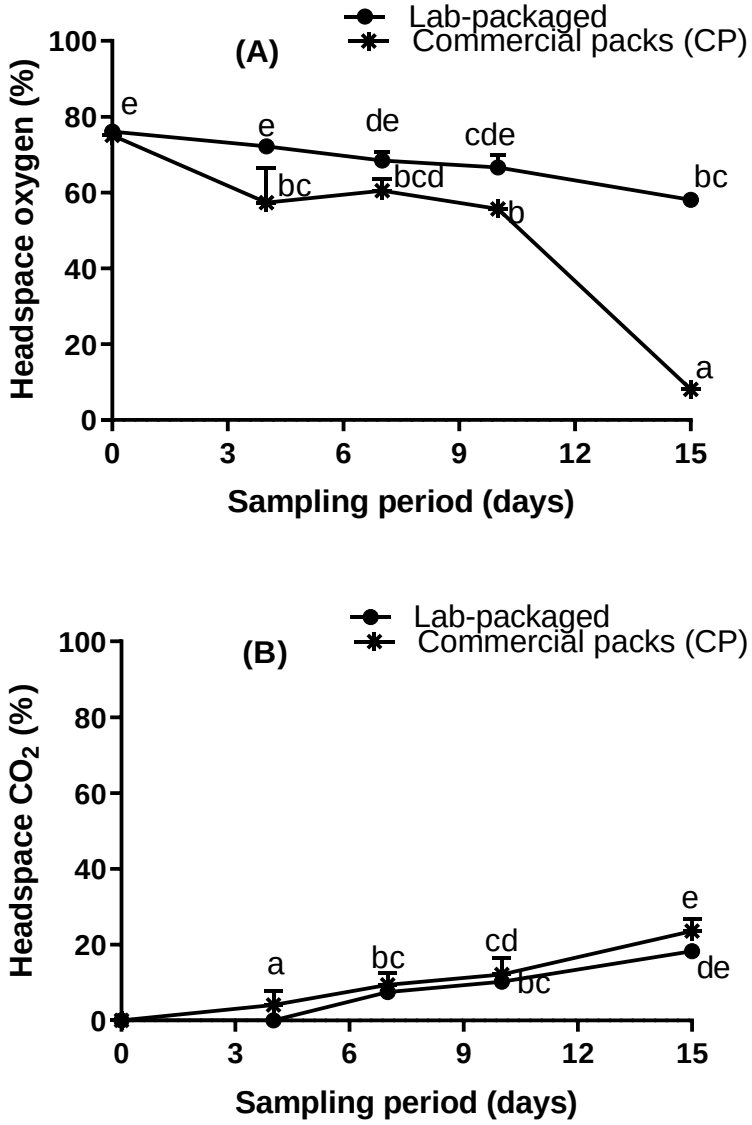


**Figure 6.4:** Headspace oxygen (A) and $CO_2$ (B) Lab-packaged mussels and Commercial packs (CP) stored at 4°C stored for 15 days. Results represent mean and standard deviation of triplicate (n=3). Different superscript denotes significant difference ($p < 0.05$) in the means of $O_2$ or $CO_2$.

### 6.3.3 Mortality

No mortality was observed on day 0 in both the treatment and the Commercial packs (CP) (Figure 6.5). A significant increase in mortality was observed in both from day 4 onwards. No significant difference ($p>0.05$) in the mortality was observed in the Commercial packs (CP)on days 4, 7 and 10, but there was a significant increase ($p<0.05$) in mortality of the

Commercial packs (CP)from 25% to 71% on day 15. The Lab-packaged treatment showed a significant increase in mortality between days 4 and 10 (11% to 40.12%) but was subsequently lower (18%) on day 15.

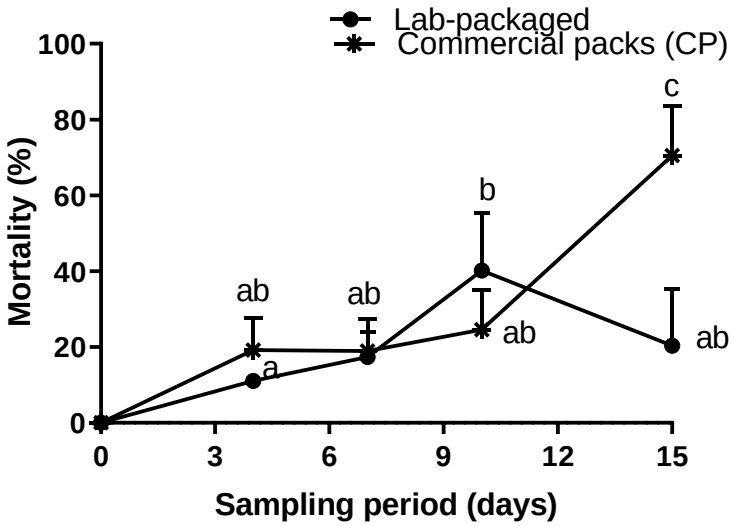


**Figure 6.5:** Mortality (%) of Lab-packaged mussels and Commercial packs (CP) stored at 4˚C stored for 15 days. Results represent mean and standard deviation of triplicate (n=3) samples. Different superscript denotes significant difference ($p < 0.05$) in the means of mortality.

### 6.3.4 Physicochemical analysis

The pH of pouch water of Commercial packs (CP) was not significantly different across the storage period (Figure 6.6A). A significant difference ($p<0.05$) was however, observed in the lab packaged treatment across the storage period. The initial mean pH of Lab- packaged treatment significantly decreased from 5.90 on day 0 to 5.22 on day 4. A further significant increase to 5.67 was observed on day 7 but was stable subsequently.

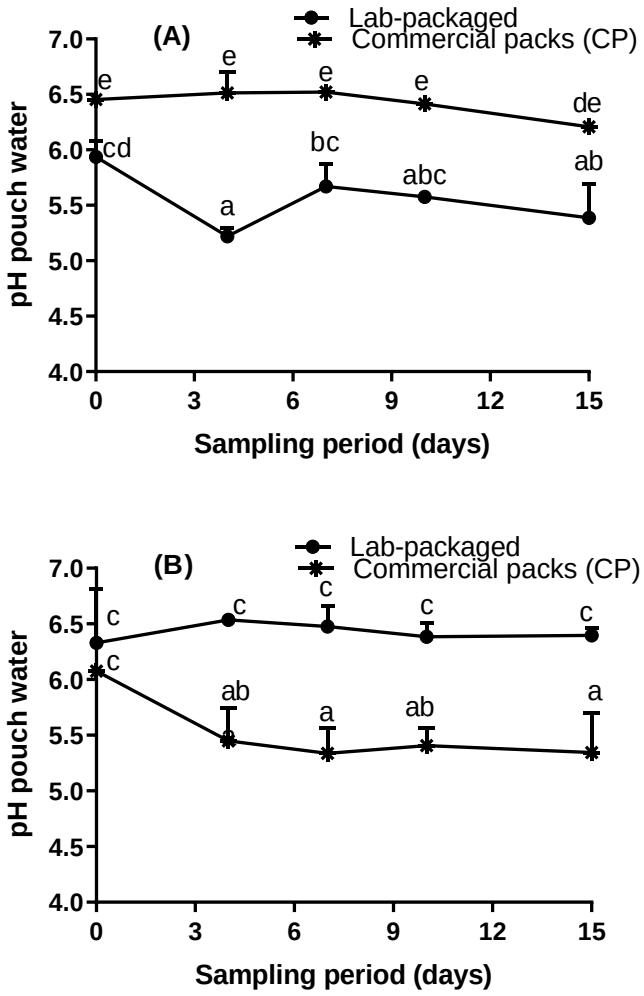


**Figure 6.6:** pH of pouch water (A) and mussel meat (B) of Lab-packaged mussels and Commercial packs (CP) stored at 4˚C stored for 15 days. Results represent mean and standard deviation of triplicate (n=3) samples. Different superscript denotes significant difference ($p < 0.05$) in the means of pH.

Unlike pouch water, the pH of mussel meat of Lab-packaged treatment was not significant different ($p>0.05$) across the storage period at a pH = 6.4 (Figure 6.6B). A decrease in the pH of mussel meat of Commercial packs (CP)treatment was observed on day 4 from 6.08 on day 0 to 5.45, but it remained stable subsequently. At the onset of storage (day 0), there was no significant difference ($p>0.05$) in the pH of mussel meat of Lab-packaged (6.33) and

Commercial packs (CP) (6.07) treatments. However, from day 4 onwards the pH of the Commercial packs (CP) meat (5.45) was significantly lower than that of the Lab-packaged treatment (6.54) There was no significant difference ($p>0.05$) in the TAN detected on day 0 of both treatments (2.5 mg $mL^{-1}$ – 2.8 mg $mL^{-1}$), and both were below tolerance limit of blue mussels (Figure 6.7). A significant decrease in the TAN of Lab-packaged treatment was observed on day 4 (to 0.58 mg $mL^{-1}$) and thereafter it further declined to be undetectable on day 10 and 15. TAN in the Commercial packs (CP) also decreased significantly, but not until day 7 (0.75 mg $mL^{-1}$) from 3.17 mg $mL^{-1}$ on day 4. Thus, TAN decreased during storage for both the treatment and the Commercial packs (CP) but did so more slowly in the Commercial packs (CP).

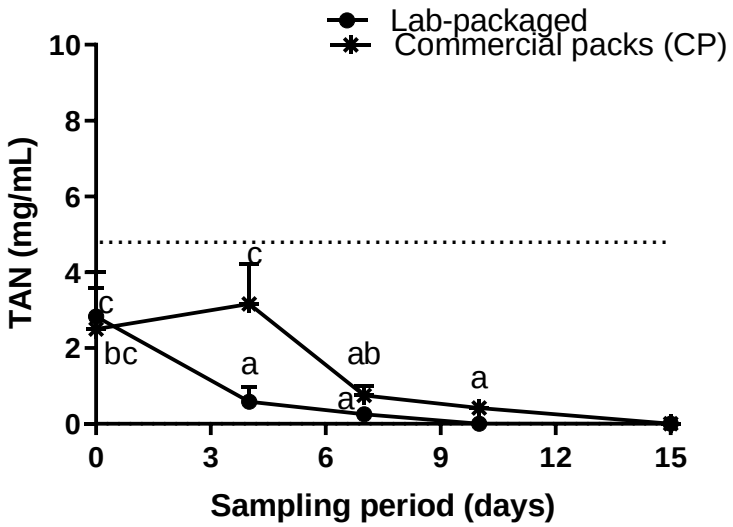


**Figure 6.7:** Total ammonia nitrogen (TAN mg $mL^{-1}$) of Lab-packaged mussels and Commercial packs (CP) samples stored at 4˚C stored for 15 days. Results represent mean and standard deviation of triplicate (n=3) samples. Broken line represents tolerance limit.

### 6.3.5 Microbiological analysis

There was no significant difference ($p>0.05$) in the mean TVC of pouch water observed between treatment and Commercial packs (CP)across the storage period (5.12 – 8.16 log CFU $mL^{-1}$) (Figure 6.8A).

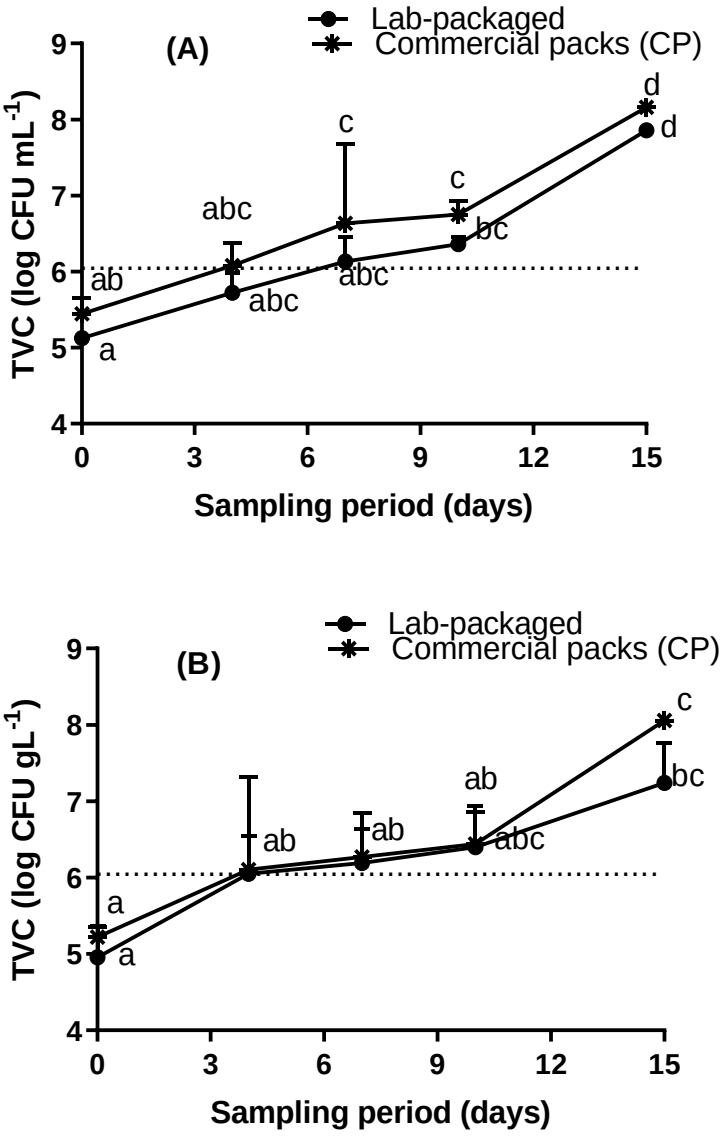


**Figure 6.8:** Total viable count (TVC) of pouch water (A) and mussel meat (B) of Lab-packaged mussels and Commercial packs (CP) stored at 4˚C stored for 15 days. Results represent mean and standard deviation of triplicate (n=3) samples. Broken line represents acceptable microbial limit. Different superscript denotes significant difference ($p < 0.05$) in the means of TVC.

The mean TVC was within the acceptable microbial limit on day 0 and day 4 in Lab-packaged treatment but was slightly above in the Commercial packs (CP). There were increase in the mean TVC in both on day 7 (6.13 – 6.63 log CFU $mL^{-1}$) and subsequently.

No significant difference ($p>0.05$) was observed between the TVC of mussel meat of the treatment and the Commercial packs (CP) on each day of the storage period (Figure 6.8B). There was a significant increase in TVC in both the treatment and the Commercial packs (CP)on day 15 compared to day 0.

### 6.3.6 Meat condition index

There was no significant difference ($p>0.05$) in the meat condition of treatment and Commercial packs (CP) across storage days (38.92 – 40.09%) (Figures 6.9). The mean MCI of treatment was above marketable meat yield (30%) throughout the storage period. The mean MCI of the Commercial packs (CP) was slightly below marketable value on day 7, but with a wide variation in the replicates. The mean MCI was above the marketable value subsequently.

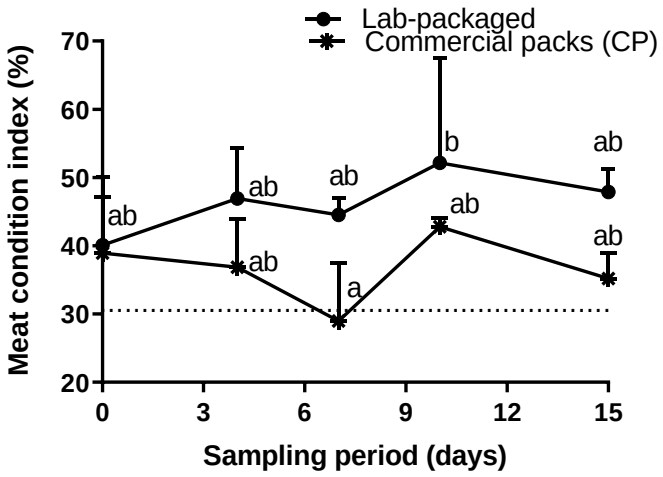


**Figure 6.9:** Meat condition index of Lab-packaged and Commercial packs (CP) samples stored at 4˚C stored for 15 days. Results represent mean and standard deviation of triplicate (n=3) samples. Broken line represents marketable meat condition. Different superscript denotes significant difference ($p < 0.05$) in the means of meat condition index.

### 6.3.7 Correlation of QI and quality parameters

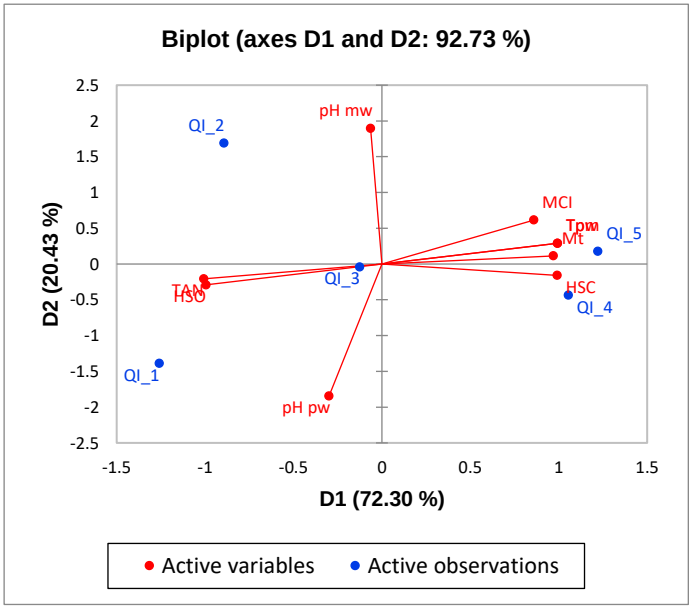


**Figure 6.10:** Principal component analysis (PCA) of the quality parameters of MAP live mussels stored at 4°C for 15 days. QI = Quality index, (QI_1 = QI for day 0, QI_2 = QI for day 4, QI_3 = QI for day 7, QI_4 = QI for day 10 and QI_5 = QI for day 15), Mt = Mortality, pH pw = pH pouch water, pH mw = pH mussel meat, Tmm = TVC mussel meat, Tpw = TVC pouch water, HSO = Headspace oxygen, HSC = Headspace $CO_2$, TAN = Total ammonia nitrogen and MCI = Meat condition index.

It was observed from the PCA that some of the quality parameters had positive or negative influence on the QI of the mussels (Figure 6.10). For example, it was observed that TAN and HSO are negatively correlated with QI unlike MCI, Tpw, Mt, Tmw and HSC that were positively correlated with QI. However, pH was marginal. The pH of mussel meat positively influenced QI while the pH of pouch water negatively influenced the QI. As expected, it was also observed that high initial headspace oxygen was associated with good quality as indicated by low QI values. However, the headspace concentration of oxygen declines with storage due to consumption and this was directly related to decline in the quality of the mussels. This was indicated by increased QI. In addition, high values of mortality, microbial counts (TVC) and $CO_2$ are correlated with high QI and poor quality. TAN was negatively correlated with QI. This was because TAN was low on day 0 and zero on day 10 and 15. Hence, microbial consumption of TAN occurs more faster than its production. MCI remained high and above the minimum acceptable level. Microbial metabolism (increased numbers, increasing $CO_2$, decreasing $O_2$) is correlated with decreasing quality of the mussels.

## 6.4 Discussion

### 6.4.1 Development of QIM tool

QIM tools have been developed for seabream (Alasalvar et al., 2001), spiny lobster (Goncalves et al., 2015), head-on and gutted (HOG) Atlantic salmon (Churchill et al., 2016). However, no such tool has been developed for mussels or bivalve shellfish. Therefore, this study aimed to develop a QIM that can be used for the assessment of the freshness and shelf-life prediction of live mussels.

According to Borges et al. (2013), the shelf-life of seafood is influenced by both intrinsic and the environmental factors and can be estimated based on the attributes of the seafood. It is the period in which the quality attributes of seafood remained acceptable (Ritter et al., 2016). The result of this study showed that the quality attributes of the mussels deteriorated over storage time. The end of shelf-life of MAP live mussels in this study was determined when the quality attributes deteriorated, and the mussels were rejected by the assessors. This was similar to the study of Borges et al. (2013) that reported the quality attributes of spiny lobster stored on flake ice deteriorated with storage.

In this study, a total of 21 demerit points for live mussels were obtained. Teixeira et al. (2009) reported 22 demerit points with 11 parameters for *Micropogonias furnieri* stored on ice for 18 days and obtained a shelf-life 14 day. Li et al. (2017) turbot stored at 4°C for 18 days and obtained 20 demerit points comprising of 5 attributes and 9 parameters with

the shelf-life estimated to be 15 days. A total of 19 demerit points, 3 attributes, 7 parameters and shelf-life of 14 days were reported for Mediterranean hake (*Merluccius merluccius*) stored at 4°C (Baixas-Nogueras et al., 2003). *Eucinostomus gula* stored on ice for 18 days was also reported to have 3 attributes, 9 parameters and 10 days shelf-life (Goncalves and Soares, 2017). They all reported change in attributes of the seafood with storage and production of objectionable smell on end of shelf-life. However, no published data on QIM for MAP live mussels was available.

Demerit points from 0 - 3 were assigned to individual quality attributes and parameters (Bonilla et al., 2007). The rejection day (day 10) obtained in this current study was similar to rejection time of raw whole spiny lobster stored on flake ice as reported by (Goncalves et al., 2015). As observed in the QIM developed for fish (Bernardi et al., 2013; Cyprian et al., 2008), the QI score for each parameter increases with storage days with QI = 0 signifying freshness.

It was observed that QI linearly increased with the storage time from 0 on day 0 to 19 on day 15 with high coefficient of determination ($R^2$) = 0.9456 which indicates a good fit of the data as expressed by the linear equation y (QI) = 1.261 x storage days -1.6789. The linear increase and high $R^2$ observed in this study have also been reported in other QIM studies. For example, Ritter et al. (2016) developed a QIM for gutted ice-stored hybrid tambatinga and reported QI = 0.483 x storage days + 1.78 ($R^2$ = 0.919), Billar dos Santos et al. (2014) developed a QIM for gutted Acoupa weakfish (*Cynoscion acoupa*) and reported QI = 1.5884 x storage days - 2.5029 ($R^2$ = 0.9897), Goncalves et al. (2015) also developed a QIM for

spiny lobster (*Panulirus argus*) and reported QI = 0.8848 x storage days + 0.6363 ($R^2$ = 0.922) and Li et al. (2017) likewise developed a QIM for turbot (*Scophthalmus maximus*), QI = 1.175 x storage days - 0.395, ($R^2$ = 0.988).

It should be noted that only 3 assessors were used in this study which was the minimum assessors required for the assessment of freshness of whole fish (Martinsdóttir et al., 2001). Increasing the number of assessors will improve the precision of the tool in predicting the shelf-life of live mussels (Sveinsdóttir et al., 2002).

### 6.4.2 Correlation between QI, microbiological quality, physico-chemical characteristics and mortality

The low values of QI observed at the onset of storage was associated with the initial high (80%) head space oxygen. However, the oxygen declined over time due to consumption by the mussels. This decline in the head space oxygen is also associated with increased QI. The high values of microbial counts (Tpw and Tmw), mortality, and $CO_2$ also correlated with high QI and poor quality of the mussels. The influence of some the quality parameters such as TAN and MCI could not be explained. For example, although TAN was low on day 0, unexpectedly, TAN was not detected on day 10 and 15. This influenced the negative correlation observed. This could be because the consumption of TAN was faster that its production towards the end of shelf-life (days 10 to 15). Similarly, the MCI was observed to be high above the minimum acceptable level throughout the storage period.

Microbiological (TVC), physicochemical, and sensory characteristics are usually evaluated to assess the quality of seafood (Ritter et al., 2016). In this study, a direct correlation was observed between the quality attributes, microbiological (TVC), physico-chemical characteristics and mortality. At the onset of storage, the of TVC both mussel meat and pouch water were below acceptable microbial limit (6 log CFU $g^{-1}$ or $mL^{-1}$). This was because the mussels were freshly harvested. However, when mussels die, the innate immune system collapsed and the mean TVC increased because dead mussels provided suitable medium for microbial proliferation. Microbial degradation and autolytic breakdown of the tissues of dead mussels contributed to the objectionable smell and eventual rejection of the mussels as observed in this study.

As reported in Chapters 2 and 4, pouch water contributed to the increased TVC. Bernardez and Pastoriza (2011) reported 4.18 log CFU $g^{-1}$ in mussel meat from high initial oxygen packed live mussels stored > 4°C. As the storage days increased, accumulation of organic waste increases which also increased the rate of mortality thereby reducing the quality and shelf-life of the MAP live mussels. This was like the observation of Bernardez and Pastoriza (2013). They observed an increase in accumulation of organic waste that, coupled with decomposed mussels, caused an increase in TVC of mussel meat of live mussels stored at 7°C. The growth and metabolism of such spoilage bacteria resulted in production of objectionable smell which is easily perceived when the packs were opened thereby leading to rejection of the palatability of the mussels. No mortality was observed in the treatment and Commercial packs (CP)on day 0 because mussels were freshly packed without stress.

However, mortality started on day 4 at 11% in Lab-packaged and 24 % in the Commercial packs (CP) samples which is more than 20% mortality threshold of live mussels packed in 75 and 85% oxygen and caused by accumulated by-products and metabolites leading to rejection as proposed by Bernardez and Pastoriza (2011).

Since the mussels were in a closed system, ammonia could not be removed from the pouch thereby accumulating with storage days. However, a significant decrease in TAN was observed in both treatment and Commercial packs (CP) as storage days increased. The reason(s) for this observation could not be ascertained as this was different from the results obtained in other Chapters. According to Barrento et al. (2014), ammonia also contributed to the objectionable smell due to microbial breakdown to ammonia. In this current study, the mean TVC in the treatment and Commercial packs (CP) was above acceptable microbial limit on day 10 which was the commercial shelf-life day.

Meat condition index (MCI) is used to indicate the marketability of shellfish. In this study, the MCI was observed to be above acceptance limit (30%) at the start of storage. The MCI of Commercial packs (CP) samples was however, below acceptance limit on day 7. Stress and any energy demanding situations such as spawning have been identified as major factors contributing to low MCI (Orban et al., 2002) but the reduced MCI on day 7 observed in the CP used in this study could be due to sampling variation as triplicate individual samples were used. More so, the CP were packed earlier (8 hr) compared to the lab-packed samples. Mussels used in this study were not spawning at the time of experimentation.

## 6.5 Conclusions

A QIM tool that adapts existing QIM concept but applied in a different context for the assessment of the quality of MAP live mussels was developed in this study. The tool is easy, fast and could be a reliable assessment of the freshness of live mussels and other shellfish such as oysters. The QIM tool consisted of 3 quality attributes, 7 parameters with 28 descriptors and 21 demerit points. The QIM tools could be used by both industry and consumers. Microbial metabolism in terms of increased microbial counts, oxygen consumption and increased production of $CO_2$ influenced the quality of mussels. Further experimental study is required to apply the developed tool for evaluation of freshness of raw (shucked) mussel packaged at different headspace oxygen and storage temperatures. There is need to investigate its usefulness to evaluate live mussels on ice versus packed in high oxygen. Similar study is also required on MAP live mussels packaged at different headspace oxygen.

# Chapter 7: Significance of study, general discussion, limitations, future research and conclusion

## 7.1 Introduction

Mussels are sold as live, frozen or processed seafood (Fernandez et al., 2015; Hicks, 2016). Like other seafood, mussels are perishable and susceptible to microbial spoilage (Briones et al., 2010). Modified atmosphere packaging (MAP) as a hurdle technology involves the deliberate exchange of gas in a product with the sole purpose of improving the shelf-life (Udayasoorian et al., 2017). Application of modified atmosphere to extend the shelf-life of live products is a development that requires improved mechanistic understanding. Shelf-life and distribution are limiting factors in exploiting live mussels as premium seafood. Live mussels can be distributed with added oxygen in a sealed consumer pack but mussels that die will be subject to increased microbial degradation. Biological processes within the mussels' tissue can also produce a fishy odour limiting consumer acceptance.

The main objective of this study was to describe the spoilage mechanisms that limit the shelf-life of MAP live mussels. Understanding the spoilage mechanisms potentially enables the identification of means to ameliorate them with a view to extending shelf-life. Specifically, this study investigated:

- The impact of depuration time, headspace oxygen and storage temperature on the shelf-life of freshly harvested live mussels **(Chapter 2).**
- The identification of volatile metabolites as indicators of freshness and spoilage in the headspace of MAP live mussels using headspace-solid phase micro-extraction (HS-SPME) **(Chapter 3).**

- The microbial community succession in MAP live mussels stored 4°C for 15 days **(Chapter 4).**
- The spoilage potential and headspace volatile metabolites of a hydrogen sulphide-producing bacterium isolated from live mussels **(Chapter 5).**
- The development of a quality index method (QIM) tool for evaluation of freshness quality and the shelf-life of live mussels **(Chapter 6).**

To minimise the impact of both environmental and intrinsic factors, freshly harvested, standard sized mussels (65 – 80 mm length) with an average age of 18 ± 2 months were consistently used throughout the period of this study. The mussels were washed and cleaned with seawater, stored below 5°C and chilled with ice prior to transporting to the laboratory. Microbiological techniques, post-harvest processing technology, food technology, food chemistry and molecular biology skills were interconnectedly used at pre-pack processing and MAP processing in this study. Understanding the actual mechanisms of spoilage of MAP live mussels helps to design further experimentation that can help extend shelf-life and prevention or reduction of spoilage.

## 7.2 Major outcomes and significance of study

### 7.2.1 Optimisation of post-harvest storage conditions (Chapter 2).

The results obtained indicated that the storage of MAP live mussels at 4°C in 80% initial headspace oxygen was the most effective combination in terms of shelf-life. Four degrees

reduced microbial growth rates without mortality and with 80% initial headspace oxygen mussels showed lower metabolic activity than those packed at either 60% or 100% as seen by lower excretion of total ammonia nitrogen (TAN). The last phase of this study showed that 8 hr depuration of harvest mussels was more practical (cost) and effective when compared to other treatments (4 and 12h). To preserve the freshness and quality of MAP live mussels until consumption, they should be depurated for 8 hr, packed with 80% $O_2$ and 20% $N_2$ and stored at 4°C. Hence, these conditions were used in subsequent experiments.

### 7.2.2 Freshness and spoilage indicators (Chapter 3).

This chapter evaluated microbial quality (TVC), mussel health, freshness and spoilage volatile organic compounds (VOC) produced during storage of MAP live mussels at 4°C. The shelf-life of commercial product (CP) samples was found to be 7 days based on the microbial count and mussel mortality. Undepurated mussels also had a shelf-life of 7 days while that of depurated samples was 10 days. The 65 VOC identified in this study were grouped into alcohols, aldehydes, phenols, furans, ketones, esters, organic acids, aromatic hydrocarbons, alkanes and nitrogen- and sulphur-containing compounds. The result of multivariate analysis showed that 12 compounds contributed to freshness while 15 compounds contributed to spoilage. The freshness volatiles such as hexanal, heptanal, and octanal decreased with storage and spoilage volatiles such as dimethyl trisulphide, decanal, phenol and octadienol increased with storage. VOC can be used to monitor the spoilage of MAP packaged mussels. The VOC produced during the storage of MAP live mussels could firstly originate from spoilage bacteria, especially from the pouch water as it provides a

biological medium for microbial growth. VOC also originated from microbial degradation of mussel tissue as they were growing on mussel tissue, using it and could be contributing to the mortality of the mussel. Since bacteria contributed most to the spoilage of the mussels and that the environmental storage condition of high or low oxygen concentrations could determine which group of bacteria thrived, 16S rRNA amplicon sequencing was then used to study the microbial community succession in both mussel meat and pouch water during storage for 15 days.

### 7.2.3 Microbial spoilage profiling of MAP live mussels stored 4°C for 15 days (Chapter 4).

Microbial communities differed among the 3 treatments during storage. At the phylum level, the mussel meat of all the treatments started with high microbial diversity on day 0. Fewer and more closely related phyla were observed on days 7 and 10 in undepurated and depurated mussels. Spoilage microbiota became predominant mostly between day 7 and 10. The spoilage microbiota of commercially packed mussels was high compared to the other treatments. Day 15 had the lowest microbial diversity in all the three treatments. At the genus level, less microbial diversity was observed in all the treatments on day 0. Days 7 and 10 had a more diverse microbial community while the community was reduced in diversity on day 15 in the mussel meat. In the pouch water at phylum level, microbial diversity was high in undepurated mussels, but low in depurated and commercially packed mussels on day 0. In all the treatments, day 7 and 10 had fewer phyla compared to day 0 but increased on day 15. At the genus level, both undepurated and depurated mussels

started with highly diverse microbial communities, but this reduced on days 7 and 10. In commercially-packed mussels, diversity increased from day 0 to day 7. Proteobacteria, Cyanobacteria and Firmicutes were the three major phyla observed in the treatments (depurated and undepurated) and commercially packed mussels (control). Cyanobacteria were dominant in the mussel meat of depurated and undepurated mussels at the start of storage while Proteobacteria were dominant in the pouch water and mussel meat of commercially packed mussels. A complete shift in the phylum was observed by the end of storage of depurated mussels with Firmicutes becoming dominant in mussel meat and pouch water. Proteobacteria dominated mussel meat and pouch water of undepurated and in commercially packed mussels, however with different dominant genera. *Shewanella* (undepurated mussels), *Acidaminococcus* (depurated mussels) and *Acinetobacter* (commercial mussels) were the major genera in mussel meat, while *Psychrobacter* (depurated and commercial mussels) and *Acinetobacter* (undepurated mussels) were dominant in pouch water. Because $H_2S$ was persistently smelt in spoiled packs as observed in Chapters 2 and 3, *Shewanella* - an $H_2S$ producing bacterium isolated from live mussels - was investigated for spoilage potential in Chapter 5.

### 7.2.4 Spoilage potential and volatile metabolites produced by $H_2S$-producing bacteria (Chapter 5).

The aims of this Chapter were to isolate specific spoilage bacteria from MAP live mussels and evaluate the isolates for microbial spoilage indices using qualitative methods and volatile metabolite production. Forty-six hydrogen sulphide-producing bacteria were isolated and evaluated for trimethylamine n-oxide (TMAO) reduction, proteolytic and

lipolytic activities and hydrogen sulphide production. Twenty-eight isolates were obtained from pouch water and 18 from mussel meat. All the isolates could produce $H_2S$ on Iron agar at 25°C while 30/46 produced $H_2S$ at 4°C and tolerated 0 – 6% NaCl. Only four (4/46) isolates could not hydrolyse mussel protein. Over 80% of isolates reduced TMAO to TMA in 3 days. Results of this study showed that $H_2S$-producing bacteria isolated from MAP live mussels are good microbial spoilage indicators. The isolate with highest enzymatic activities and $H_2S$ production was identified as *S. baltica* via 16S rRNA gene analysis. An axenic culture of the isolate was inoculated into sterile mussel broth and stored at 4°C for 10 days. Forty-four VOC were identified in the sample after 10 days while 27 compounds were identified at the time of inoculation among which 19 were still present in the samples on day 10 but at reduced abundance. Groups of compounds identified were alcohols, aldehydes, phenols, furans, ketones, esters, organic acids, aromatic hydrocarbons, alkanes and nitrogen- and sulphur-containing compounds. The new VOC observed on day 10 include pentadecane, dimethyl trisulphide, octadiene, bis (dimethylethyl) benzene, methyl-phenol, ethyl benzoic acid, butylester, 3,5-octadiene, ethyl-benzene, nonanal, 2-ethyl-5-methylfuran, 3-ethylthiophene, undecane, nonanone, tetradecanal, decanal, xylene, indole, heptadecane, octadienol, benzaldehyde, 3-methyl butanol, pentyl furan 2, thiohexene and tetramethylpentane. Dimethyl trisulphide, nonanal, decanal, phenol, pentyl furan 2 and octadienol were spoilage VOC produced indicating that the off-odour observed was a combination of VOC and not just a single sulphide compound. The data from Chapters 2 to 5 were then considered in total to develop a quality index tool that can be used to evaluate quality and thus estimate the shelf-life of live mussels.

### 7.2.5 Development of quality index method (QIM) tool (Chapter 6).

A draft QIM tool that consisted of 3 quality attributes and 7 parameters with 28 descriptors and 21 demerit points was developed and a linear model was obtained (QI = 1.261 x storage days - 1.6789, $R^2$ = 0.9546, n = 3). The estimated shelf-life of MAP live mussels was projected to be 10 days with optimised duration of depuration, headspace oxygen and storage temperature, which correlated with the shelf-life observed in Chapter 2. The microbial counts were above acceptable microbial limit of 6 log CFU $g^{-1}$ or $mL^{-1}$ between days 7 and 10 when the smell was more obvious/stronger as obtained in Chapters 2 (Figures 2.13 and 2.22) and 3 (Figure 3.7). This corroborated with the microbial diversity in the mussel meat observed between days 7 and 10 in Chapter 4, along with increased mortality and decreasing oxygen. This tool could be adapted for use for other shellfish such as oysters. The draft QIM developed in this study follows the following criteria as used by Bremner:

- The major difference was temperature in which Bremner used storage on ice but 4°C was used in this case because such low temperature could stress the mussels and result into early mortality that can limit the shelf-life.
- Use of assessors – Assessors were used in this study.
- Development and description of quality attributes and parameters that were used for demerit points used to score the quality and shelf-life.

## 7.3 General discussion

Bivalves such as mussels can survive out of their aquatic environment for longer periods than other seafood and due to this, they can be sold live. However, mussels can become stressed by the method of capture, processing, handling, storage temperature, water quality and physical damage during the supply chain (Barrento et al., 2013). Although the mussels maybe able to overcome some of the stressors, combinations of several stressors and poor storage conditions can shorten the shelf-life (Barrento et al., 2013). It is therefore imperative to ensure optimum storage conditions of mussels post-harvesting.

Harvested mussels are usually depurated by placing them in a tank with clean seawater for certain period to ensure purging of the animals' gut contents and other transient materials such as debris and bacteria. A 12 hr depuration period was suggested for survival and quality by Barrento et al. (2013) who investigated handling and transporting of live mussels. This is different from the duration of depuration in Australia for oysters (48 h). The duration of depuration of mussels becomes a major commercial challenge considering the economic cost of long depuration times.

Similarly, since live mussels require oxygen for survival, the use of modified atmosphere packaging (MAP) has been adopted (Pastoriza et al., 2004). Only a few studies have been carried out on the use of oxygen and MAP for freshness and quality of live mussels (Bernardez and Pastoriza, 2011, 2013; Pastoriza et al., 2004). None of these studies

optimised the initial headspace oxygen that can help maximise the freshness and quality of live mussels until consumption.

During the supply chain, live mussels are usually transported on an ice slurry to a processing plant. Processing and handling of mussels at retail and consumer levels may adversely affect mussels, particularly at sub-optimal storage temperatures. Optimizing the duration of depuration, headspace initial oxygen and storage temperature will help to keep mussels alive at their best physiological state and organoleptic characteristics until the time of consumption. Varying shelf-lives have been reported for live mussels (6 – 7 days) (Bernardez and Pastoriza, 2011, 2013; Pastoriza et al., 2004). Cool storage temperatures both slow mussel metabolism, reducing oxygen demand and reduce microbial growth thereby slowing the spoilage of mussels, especially when packed with adequate oxygen. MAP involves changing the gas environment surrounding the seafood to also slow microbial growth and oxidative reactions thereby prolonging shelf-life in mussel meat because oxygen is usually excluded while $CO_2$ is majorly used (Sivertsvik et al., 2002). However, the mechanism involving live mussels is not fully understood because live mussels require oxygen rather than $CO_2$ that is required in MAP of dead seafood such as fish fillet. High oxygen for respiration requirement but may cause rancid oxidation and support the growth of more bacteria. The use of MAP in the shellfish industry enables convenience of home preparation of the seafood (Madigan, 2014). Because the mussels are packed alive then they must be presented with sufficient oxygen to meet respiratory requirements for the time up to consumption. The increase in the rates of diffusion oxygen into pouch water helps to maintain DO (dissolved oxygen) in the pouch water. This may

limit production of anoxic zones, which likely are associated with spoilage VOC and possibility of injurious end products. This is distinctly different from MAP of meat products in which oxygen is commonly absent or minimized. As the production of total ammonia nitrogen (TAN) was lowest at 80% headspace oxygen it was concluded that this represented a useful compromise between maximising oxygen for mussel survival but reducing the metabolic activity of the animals as seen in the production of TAN. Previous studies used between 75 – 85% oxygen and stored between 1 – 6°C and achieved 6 days shelf-life (Bernardez and Pastoriza, 2011, 2013). This was shorter than the 10 days reported in this current study. Inconsistencies in the headspace oxygen and storage temperature used have been observed thereby making it difficult to optimise oxygen concentration (Bernardez and Pastoriza, 2011, 2013). The outcomes of this current study confirm a 10-day shelf-life for live mussels is feasible and possibly it may be further extended. While the optimum post-harvest storage conditions have been established, it was not clear what causes spoilage of mussels. Microbial and autolytic activities have been reported to cause spoilage of other seafood such as fish. It is difficult to categorically state the cause of spoilage of live mussels since the innate immune system of the animals can help prevent or reduce the effect of microbial attack. However, storage will inevitably stress the animals and reduce the effectiveness of mussel defence mechanisms. Both microbial and autolytic activities are likely to contribute to spoilage. Off-odour or volatile metabolites (VOC) are evidence of spoilage of seafood (Fratini et al., 2012). VOC diversity associated with fresh material have been reported as declining during storage of fish (Aro et al., 2003; Parlapani et al., 2015), shrimps (Broekaert et al., 2013) and shucked mussels (Aru et al., 2016b). Similarly, this current study of live mussels in MAP demonstrated that some VOC were

associated with freshness and these declined during storage. Sixty-five VOC were identified from 11 organic and sulphur containing compounds. The fact that depuration resulted in significantly different types of VOC suggests that microbial activities are a significant contributor to VOC, both freshness and spoilage VOC. Reducing the total number of microbes (depuration) and their growth rates (cold temperature) are thus both important for maintaining mussels' quality. While Chapter 3 of this thesis provided information on freshness and spoilage VOC, it was not ascertained which of the VOC emanated from bacteria or from degraded mussel tissue or from pouch water or all of these. Some of the VOC observed such as indole, phenol, dimethyl disulphide, dimethyl trisulphide and decanal have strong smell even in small amounts and are readily detectable by humans. Investigating the microbial community succession during storage of MAP mussels (depurated and undepurated) sought to address this question.

The microbiota in seafood could either be from the aquatic environment, microbes indigenous to the animal, from transient gastrointestinal taxa or from external sources such as contamination from handlers. Depuration is known to remove transient bacteria from bivalves, but it was not clear the types of bacteria that are being removed. In addition, studies on microbial community succession during storage of live mussels have not been carried out. At the start of storage in this study, there was high headspace oxygen available for the mussels and this favoured the observed growth of aerobic bacteria. As storage increases, especially by days 7 and 10, available oxygen becomes reduced due to consumption by mussels and microbes, and anaerobic bacteria became dominant by the end of storage on day 15. Initially, lots of Proteobacteria taxa were present, but often in

low numbers, so it appears that a subset can easily dominate during storage. The conditions of storage are fundamentally different from what the mussels experienced in the ocean as the pouch water is stagnant, fresh water as against salt water and the numbers of microbes in it become far higher than might be seen in the ocean. This will alter microbial growth and thus the community structure around mussels and limit oxygen supply to the mussels. The successful microbiota constitutes a potential source of spoilage of the mussels. Although studies on microbial quality of live mussels have been conducted, the spoilage isolates were not identified to species level (Aru et al., 2016a; Bernardez and Pastoriza, 2011, 2013; Pastoriza et al., 2004). No study has attempted to elucidate the spoilage microbiota in live mussels. Addition of pouch water to MAP live mussels may be contributing a negative rather than positive impact because it serves as biological medium for microbial growth and VOC production. Similarly, oxygen added to the packs dissolve in the pouch water thereby reducing the amount of headspace oxygen available to the mussels right from the start of storage, thus further stressing the mussels which then contributes to mortality. Mussels may also take oxygen from the pouch water, but they are in direct competition with microbes, that are likely to access dissolved oxygen first as they are outside of the mussels. Microbes therefore appear to have good access to oxygen and given their increase in numbers, may contribute substantially to mussel stress. What would mussel survival and palatability be in dry packs? There is need to examine either exclusion of pouch water or the use of an antimicrobial agent/s that are not dangerous to mussel survival to suppress microbial growth thereby prolonging shelf-life.

It has been reported that not all microorganisms found on seafood can cause spoilage but rather only few known as specific spoilage organisms (SSO) (Dalgaard, 1995b). The spoilage potential of the bacteria isolated in this study was investigated using *Shewanella* as model organism. *Acidaminococcus, Shewanella, Acinetobacter* and *Psychrobacter* were identified in this study using 16S rRNA amplicon sequencing as four major spoilage genera due to their abundance and dominance, *Shewanella* was chosen because it can easily be cultured and could grow at 25°C within 48 hr and a lot of literature have associated it with seafood spoilage. For example, Ge et al. (2017) associated it with the spoilage of fish stored at 4°C. It was also reported in the spoilage of half-shell Pacific and Sydney rock oysters stored at 4°C (Madigan et al., 2014). A distinct black pigmentation due to precipitation of iron sulphide (FeS) on agar makes it easy to identify phenotypically (Parlapani et al., 2017). All the isolates in this study were identified as *Shewanella baltica*. Among the *Shewanella*, both *S. putrefaciens* and *S. baltica* have been associated with spoilage of seafood (Zhu et al., 2017) such as fish (Ge et al., 2017; Jorgensen and Huss, 1989; Parlapani et al., 2017). Sterile cooked mussel juice was used in this study of spoilage potential as in previous studies (Parlapani et al., 2017) because of the difficulties in using raw seafood which has many other taxa present (Dalgaard, 1995b; Dehaut et al., 2014; Parlapani et al., 2017). The six spoilage VOC (dimethyl trisulphide, nonanal, decanal, phenol, pentyl furan 2 and octadienol ) identified in this study from *S. baltica* have also been identified in previous studies involving spoilage of *S. baltica* in fish. For example, Parlapani et al. (2017) reported production of nonanal and decanal by *S. baltica* inoculated into sterile fish juice agar that was incubated at 0 and 15°C for 18 days.

The spoilage of seafood involves several processes, likewise the assessment of spoilage involves use of physical and olfactory methods. These methods have been used for assessing the freshness of seafood such as fish, however, both methods can be subjective (Bernardi et al., 2013).

The use of a quality index method (QIM) was proposed as better means of assessing the freshness of fish because it combines both physical assessment and olfactory methods (Billar dos Santos et al., 2014). No such method has been developed for live mussels. This quality and freshness assessment method was therefore developed in this study. A strong relationship among changes in the attributes of both raw, cooked mussel meat and pouch water was observed.

For example, as raw mussel meat became very soft especially on day 10, this was also observed in the cooked meat while the pouch water became brown. Similarly, it correlated with the TVC, mortality, reduced oxygen and production of VOC indicating that reduced oxygen was associated with mortality. As mussels died their flesh provided more biological medium for microbial growth and production of VOC. These were more obvious on day 10. Therefore, the end of shelf-life for depurated mussels was designated to be day 10. This study shows that depuration reduces the numbers of microbes and microbial diversity, thus delaying spoilage. Pouch water has much higher numbers of microbes more quickly than does meat. Thus pouch water appears to be a major cause of spoilage. High headspace oxygen is necessary for preserving mussels, but is also able to enhance growth of aerobic

microbes - this occurs mainly in the pouch water as evidenced by wider diversity of taxa present in it.

The initial taxa were representative of indigenous marine microbes (e.g Cyanobacteria - *Synechococcus* and Proteobacteria). The low numbers but wide divesity of Proteobacteria provide a good foundation for opportunistic spoilage organisms to grow rapidly. High numbers of microbes in the pouch water could restrict oxygen supply to mussels because diffusion is relatively inefficient and microbes, being on the outside of the mussels, get first use of the oxygen.

Similarly, TAN may accumulate mussels get more stressed. These factors both increased microbes and more stressed mussels in the decline of fresh VOC and rise of spoilage VOC as well as in mortality. Some spoilage VOC such as dimethylsulphide (DMS), could be from the breakdown of algal osmolytes which ties in with Cyanobacteria being initially dominant. The dominance of anaerobes like *Acidaminococcus* in spoilt mussels was observed. These are likely to be endpoints and may be indicative of spoiled mussels rather than mussels that are in the process of spoiling. *Acidaminococcus* was found in high numbers in the mussel meat than in pouch water. This was because availability of oxygen to mussels is limited by poor diffusional transport through the pouch water and obligate anaerobes were likely to grow in hypoxic or anoxic microzones around the meat. The end result was that - with pouch water, 10 days is the maximum length of shelf-life.

Factors contributing to the spoilage of live mussels at pre-harvest and packaging includes mussel condition, mussel damage, indigenous microbiota and organic loading. On day 0, the pouch water of the mussels packed with 60% oxygen (commercial packs) had freshness VOC and indigenous microbiota in low numbers although diverse. Aerobic and facultative bacteria were favoured. On days 7 and 10, indigenous microbiota increased while diversity decreased, facultative and anaerobic bacteria were favoured. Increase in the organic loading from morbid or dead mussels resulted in increased growth of indigenous microbiota. As storage days increased (day 0 to day 15), the respiration of healthy mussels and microbes in the pouch water resulted in decreased oxygen which changes the microbial community in the pouch water and mussel meat, thereby increasing stress in the mussels and increase in mortality. At the onset of storage (day 0), freshness VOC was high but decreased across the storage days, while spoilage VOC was initially low but increased with storage days. In order to increase the shelf-life of MAP live mussels, duration of depuration needs to be increased. This will help to decrease indigenous microbiota and organic load. Decreased storage temperature will increase lag phase and decrease total oxygen consumption. The headspace oxygen should be increased to 80%. This will increase available oxygen to the mussels to maintain respiration and production of moderate TAN. Hence, improved processing with decreased temperature increases lag phase and decreases growth rates. The microbial mechanisms of spoilage of MAP live mussels with pouch water in the commercial (60% headspace oxygen) and laboratory packs (80% headspace oxygen) are summarised in Tables 7.1 and 7.2 below.

**Table 7.1:** Summary of the spoilage parameters in 60% headspace oxygen MAP live mussels with pouch water in commercial packs.

| Parameters | Storage period (days) | | | |
|---|---|---|---|---|
| | **0** | **7** | **10** | **15** |
| **Mussel quality** | Good | Good, low mortality | Poor, high mortality | Very bad, high mortality |
| **Microbiota in pouch water (TVC)** | Low (< allowed) | Increased (≥ maximum allowed) | > maximum allowed | >> maximum allowed |
| **VOC** Freshness | Present | Present (decreased) | Absent (≈ 0) | 0 |
| Spoilage | Absent/low | Increased (some) | Present | Present (High) |
| **Oxygen (%)** | 60% | Decreased to 42% | 4% | 2% |
| **TAN** | Below limit of detection (BLD) | Increased (6 mg $mL^{-1}$) < maximum allowed (5 mg $mL^{-1}$) | Increased (7 mg $mL^{-1}$) | Decreased (3 mg $mL^{-1}$) |

**Table 7. 2:** Summary of the spoilage parameters in 80% headspace oxygen MAP live mussels with

| Parameters | Storage period (days) | | | |
|---|---|---|---|---|
| | **0** | **7** | **10** | **15** |
| **Mussel health** | Good | Good, no mortality | Good, low mortality | Poor, high mortality |
| **Microbiota in pouch water (TVC)** | Low (< allowed) | low allowed | > allowed (reduced) | ≥ maximum allowed |
| **VOC** Freshness | Present | Present (decreased) | Present (low) | Absent (≈ 0) |
| Spoilage | Absent | Low | Present | Present (High) |
| **Oxygen (%)** | 80% | Decreased to 72% | Decreased to 67% | Decreased to 62% |
| **TAN** | Below limit of detection (BLD) | < 5 mg $mL^{-1}$ | < 5 mg $mL^{-1}$ | BLD |

pouch water in laboratory packs.

## 7.4 Limitations of the study

This study has some limitations such as:

1. The smaller number of mussels in each laboratory packs (6 – 8 mussels) compared to > 40 mussels in the commercial packs (Chapter 2 – 6). Repeating experiments under commercial conditions would be the next step.
2. In the MCI sections (Chapters 2, 3 and 6), the mussels were not tasted. Hence it was difficult to ascertain the palatability fully. Instead this relied on smell only.
3. The optimized duration of depuration (Chapter 2) was not tested in industrial settings to determine commercial practicality. Similarly, on day 15 when the pouch water became slurry, TAN could not be detected by the method which may not necessarily mean absence. Hence, there is need for use of other methods such as the use of spectrophotometer.
4. In Chapter 3, there were no control standards of volatiles to help ascertain the identified VOC by comparing the retention times with those of corresponding authentic compounds.
5. In Chapter 4, there is need for repeated studies using multiple packs to verify the pattern of microbial succession that was observed. For example, different dominant microorganisms were observed in the 3 treatments (*Shewanella* - in undepurated mussels, *Acidaminococcus* in depurated mussels and Acinetobacter in commercial mussels). Furthermore, although similar treatments gave similar shelf-

life outcomes there were differences noted in the environmental and microbial factors which could be examined further.

6. In Chapter 5, the use of HS SPME could be too expensive for the use of industry. The VOC of uninoculated mussel juice broth (MJB) used as control was not tested. This would have helped to ascertain if the VOC reported in the inoculated MJB was produced by *Shewanella* or just oxidation by products.
7. In Chapter 6, mussels were not stored on ice as done by Bremner (1985) thus an amended protocol was needed which really needs more validation for commercial use. Similarly, only three assessors were used. There is need for repeating the study to validate the QIM tool used in this study. Thus, the strength of the data could be enhanced and validated by repetition with more assessors.

## 7.5 Future research and conclusion

This study has successfully provided previously lacking information on optimising post-harvest storage conditions required for keeping the freshness of MAP live mussels that indicates the commercial shelf-life of live mussels can be increased from 7 days to 10 days. There is still need for future work that can be built on from these results. Firstly, studying the economics of the duration of depuration is recommended as such a long time (8 h) may not be commercially feasible. The different depuration durations were not tested at the industry level. This was due to time and resource limitations. The 8 hr depuration recommended only removed loosely attached microbiota – as would be expected - implying that the remaining bacteria form part of the spoilage microbiota. Only *S. baltica*

was cultured as an SSO in this study – others could also be examined in axenic culture. In addition, known mixed cultures (based on community succession in mussels during storage) could be examined to look specifically at roles in spoilage. Further study on the use of Zydox or other sanitisers as sanitizing and antimicrobial agent is worthy of consideration.

This study demonstrated use of HS SPME /GC-MS as a tool to study freshness and spoilage VOC that are evolved during storage of MAP live mussels. HS SPME/GC-MS is expensive and is not practical as a monitoring tool, thus a draft QIM was developed. The QIM tool could be directly correlated with VOC evolution thereby accurately modelling the development of spoilage. There is a need to understand the impact of microbial growth and thus of VOC from pouch water by excluding pouch water and studying the VOC produced during storage along with mussel survival and shelf-life. This current study showed that pouch water might contribute to spoilage by providing a biological medium for rapid and significant microbial growth and VOC emission. Mussels that have been depurated could be packed in MAP conditions without any pouch water (i.e. dry), although they might exude intervalvular fluids. Total viable count, mortality, microbial community change and VOC should then be examined to see if shelf-life can be extended. In this current study, it was not known if there is a spoilage gene or genes in *Shewanella* that could be identified as responsible for spoilage. Therefore, there is a need to identify spoilage gene biomarkers in *S. baltica* using gene profiling. Such a gene or genes could be used to understand other SSO present in MAP live mussels. Does *Acidaminococcus* require some

other organism to hydrolyse proteins or does it do this itself? Therefore, the synergistic and spoilage potential of *Acidaminococcus, Acinetobacter* and *Psychrobacter* could be studied.

In conclusion, this study showed that depuration of mussels at 8 hr, packing with 80% oxygen and storage at 4°C would help to retain the quality of live mussels. Similarly, there is need to treat the pouch water added to live mussels or to avoid it because pouch water serves as source of microbial growth that contributes to the reduction of the quality and to spoilage of live mussels. *Shewanella, Acidaminococcus, Acinetobacter* and *Psychrobacter* were identified as spoilage bacteria in MAP live mussels. The draft QIM tool modified from Bremner (1985) developed in this study could be used to estimate the freshness and shelf-life of live mussels. Volatiles such as hexanal, heptanal, and octanal could also be used to determine the freshness of live mussels. Hence, the spoilage mechanisms of live mussels involved ineffective depuration, reduced headspace oxygen and the pouch water. These aided mussel handling stress, microbial growth, production of VOC and eventual rejection of the products on day 10 under the storage conditions examined.

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## Appendix A

**Isolate S1** (MF59911)

ACATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAA
TGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGG
GGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGT
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CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGC
GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCTGT
TGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCC
GAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGG
GCTCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTA
GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCT
CAGGCAC

**Isolate S2** (MF599112)

ACATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAA
TGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGG
GGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGT
AATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGC
GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGTTTAATACGCTGT
TGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCC
GAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGG
GCTCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTA
GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCT
CAGGCAC

**Isolate S3** (MF599113)

ACATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAA
TGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGG
GGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGT
AATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGC
GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCTGT
TGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCC
GAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGG
GCTCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTA
GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCT
CAGGCAC

**Isolate S4** (MF599114)

CACATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTA
ATGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACG
GGGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGG

TAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCT
GTTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
CCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCC
GGGCTCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTG
TAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG
CTCAGGCAC

**Isolate S5** (MF599115)
ATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAATG
CCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGG
GAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGT
GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCTGTTG
CTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCGA
GCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGC
TCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC
GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGCAC

**Isolate S6** (MF599116)
ATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAATG
CCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGG
GAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGT
GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCTGTTG
CTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCGA
GCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGC
TCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC
GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGCAC

**Isolate S7** (MF599117)
CACATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTA
ATGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACG
GGGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGG
TAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAATAACTTAATACGTT
ATTACTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
CCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCC
GGGCTCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTG

TAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG
CTCAGGCAC

**Isolate S8** (MF599118)
TGCAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCC
TAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGA
AAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATG
GCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTG
TGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCTGTTGC
TGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCGAG
CGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGCT
CAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCG
GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG
GCAC

**Isolate S9** (MF599119)
ATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAATG
CCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGG
GAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGT
GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCTGTTG
CTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCGA
GCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGC
TCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC
GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGCACG

**Isolate S10** (MF599120)
ATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGGCGGACGGGTGAGTAATG
CCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGG
GAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGT
GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCAGCTTAATACGCTGTTG
CTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCGA
GCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGC
TCAACCTGGGAATTGCATTTCGAACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC
GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGCAC

## Appendix B

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HUMAN RESEARCH ETHICS COMMITTEE (TASMANIA) NETWORK

UTAS

10 October 2017

Dr Chris Burke
Institute for Marine and Antarctic Studies
University of Tasmania

Student Researcher: Olumide Odeyemi

*Sent via email*

Dear Dr Burke

Re: MINIMAL RISK ETHICS APPLICATION APPROVAL
Ethics Ref: **H0016651 - Improving mussel quality in Modified Atmosphere Packs to increase shelflife**

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We are pleased to advise that acting on a mandate from the Tasmania Social Sciences HREC, the Chair of the committee considered and approved the above project on 09 August 2017.

This approval constitutes ethical clearance by the Tasmania Social Sciences Human Research Ethics Committee. The decision and authority to commence the associated research may be dependent on factors beyond the remit of the ethics review process. For example, your research may need ethics clearance from other organisations or review by your research governance coordinator or Head of Department. It is your responsibility to find out if the approval of other bodies or authorities is required. It is recommended that the proposed research should not commence until you have satisfied these requirements.